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The Yale Journal of Biology & Medicine

179 **RENAL DISEASE** *John P. Peters*

191 **RED CELL MUCOPROTEINS** *John F. McCrea*

211 **PAPER ELECTROPHORESIS OF SALIVA** *Thorn Kinersly*

215 **DUAL NATURE OF ACID-FASTNESS** *J. W. Berg*

224 **CHANGING CONCEPTS IN PUBLIC HEALTH** *Ira V. Hiscock*

233 **EVENTS**

235 **BOOK REVIEWS**

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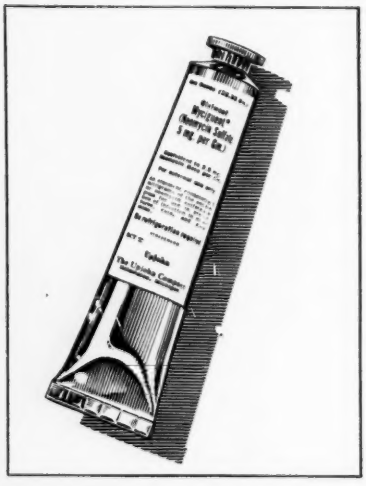

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CONTENTS

179 **SOME REMARKS ON RENAL DISEASE** *John P. Peters*

191 **STUDIES ON INFLUENZA VIRUS RECEPTOR SUBSTANCE AND RECEPTOR-SUBSTANCE ANALOGUES. II. ISOLATION AND PURIFICATION OF A MUCOPROTEIN RECEPTOR SUBSTANCE FROM HUMAN ERYTHROCYTE STROMA TREATED WITH PENTANE** *John F. McCrea*

211 **PRELIMINARY PAPER ELECTROPHORETIC STUDY OF SALIVA** *Thorn Kinersly*

215 **THE DUAL NATURE OF ACID-FASTNESS** *J. W. Berg*

224 **CHANGING CONCEPTS IN PUBLIC HEALTH** *Ira V. Hiscock*

233 **EVENTS**

235 **BOOK REVIEWS**

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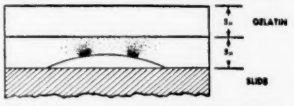

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JOHN P. PETERS | *Department of Internal Medicine, Yale University School of Medicine*

SOME REMARKS ON RENAL DISEASE*

The title of this talk is extremely broad. Obviously I cannot pretend to cover the field of renal disease in a single lecture. All I intend is to express a point of view and to suggest an attitude or approach to the subject.

Attention will be directed particularly to those diseases that lead to deterioration of the function of the kidneys accompanied by vascular disorders, especially hypertension. These entities are conventionally divided into two chief categories: those which originate in diseases of the kidney and those in which the primary disorder is arterial. The latter is in turn frequently divided into two classes: those in which there are definite morphological changes in the arteries and those in which hypertension appears without morphological arterial equivalents. There is ample experimental and pathological evidence that hypertension and vascular disease may be produced by renal disorders; there is no comparable body of data to support the concept of primary or essential hypertension and hypertensive vascular disease. What there is would suggest that its causes might be legion. The terms *essential* and *idiopathic,* applied to hypertension, are merely declarations of ignorance, labels for the scrap-basket into which are dumped all cases that have hypertension for which no obvious—and particularly no renal—origin can be found. Recognition of this fact would, I think, hasten discovery of the nature and causes of these conditions by promoting inquiry; the attachment of polysyllabic labels has throughout medical history created such mental and spiritual satisfaction that it has tended to stifle curiosity.

I cannot refrain from putting in a plea for more careful study of the natural history of disease. Though it may seem inappropriate to this advanced era, much of our knowledge of renal diseases was acquired through this channel and its potentials are not yet exhausted. I should be the last person to disparage investigations of function; but I believe that on the whole they are likely to contribute more to therapy and prognosis than to pathogenesis and diagnosis. About 1920 the ardor for classification of renal disease by an infinite number of nomenclatures based on every imaginable criterion began to cool down; or at least seemed to many thoughtful

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persons to have reached such a ridiculous state of futile confusion that they were willing, for lack of more precise knowledge, to accept a broad classification such as I have outlined above, into primary renal and primary arterial disease. The renal group was, however, smaller and the arterial group larger than it is at present, because the former consisted chiefly of glomerulonephritis. That chronic pyelonephritis could produce hypertension was admitted, but was given little weight until the next decade when it suddenly became apparent to a number of clinicians and pathologists, among them Warfield Longcope, Soma Weiss, and ourselves, that the nature of the disease, its course and clinical manifestations and its morphological consequences had not been appreciated, and that it was probably responsible, especially in women, for a large proportion of the hypertension and vascular disorders that had previously been attributed to primary arterial disease, including some toxemias of pregnancy. It was surprising to learn that in the majority of cases this disease, like congenital polycystic kidneys, though obviously originating in the kidneys, manifested itself first in hypertension and its attendant cardiovascular disorders, while obvious renal insufficiency appeared only in the terminal stages. It was also learned that the course of the disease might cover a great many years during which symptoms and signs of urinary infection might be few and trivial. These discoveries, largely a product of astute clinical observation of the course or natural history of the disease, snatched from the scrap-basket of primary vascular disease a large proportion of the cases that had previously been consigned to that repository. It also pointed the way to what I believe might be the most fruitful attack on the incidence of hypertension at this moment, the elimination of chronic pyelonephritis. Twenty years ago this was impossible because there were no effective means of combatting infections of the urinary tract. The introduction of antibiotics has completely changed the potentialities, but practice lags far behind. The most effective antibiotics are not generally carefully selected, mere symptomatic relief is too often accepted as evidence of eradication of infection, and care is not taken to search for and remove features that are conducive to recurrence of infection—incomplete emptying of the bladder and obstructive or irritative conditions in and about the urinary tract. In fact, the introduction of antibiotics seems almost to have enhanced the cavalier spirit that prevailed when there were no effective therapeutic agents. Acute pyelonephritis is regarded as a trivial matter because it can be so readily relieved. Inlying catheters, a fruitful source of chronic pyelonephritis, are inserted for esthetic reasons that may have more than a remote connection with the shortage of nurses.

The incidence of hypertension in relation to age follows a course that is often termed the curve of senescence, a term that becomes less and less acceptable as one's age advances. There is, to be sure, a bump in the teens and early adolescence that marks the major incidence of acute glomerular nephritis, and a gradual upward slope beyond this, indicating further accessions of glomerulonephritis, pyelonephritis, and other conditions that are no respecters of age. In the fifth decade, however, the curve begins to rise with increasing acceleration until depletion of the population finally retards it again. To what extent does the accelerated rise after 40 represent the introduction of a new set of disorders connected with the aging process as the term *senescence* implies, or to what extent does it represent merely results of past injuries? In other words, does it describe the aging of persons or the aging of disease? Again polycystic kidneys afford an example. Unless infection, pregnancy, or some other complication is superimposed, this condition, though congenital, usually manifests itself in hypertension and its consequences in the fifth decade; renal insufficiency is commonly delayed until the next decade. This and pyelonephritis incurred early in life, but manifested in hypertension only after 40, are not strictly signs of aging of the patient, but of aging of a disease, otherwise age would have to be estimated in terms of propinquity to death. The fact that the statistical incidence of hypertension increases with age is not satisfactory evidence that it is an essential item of the aging process. The statistical correlation of two variables does not *ipso facto* signify that either one is the cause or effect of the other. Furthermore, if the rise of the curve after 40 is due to the accession of entirely new disorders connected with the aging process, it does not follow that the incidence of disorders previously operative ceases. Nevertheless, so strong is the preconception that hypertension is a part of aging, usually implying primary vascular disease, that it is often taken for granted that hypertension is a natural, though deplorable, event in an elderly patient. The oldest acute glomerulonephritic in our series was nearly 80. There are a few beyond 50 who were consigned to the category of chronic hypertensive vascular disease with nephrosclerosis. Subsequently the signs of disease miraculously disappeared, permitting the diagnosis of acute nephritis to be made in retrospect.

If there is any advantage other than a thirst for precision in making more than a purely descriptive diagnosis, each case must be analyzed with the most open-minded and meticulous care. The difficulty of differentiating chronic pyelonephritis from primary vascular disease has already been mentioned. The clinical distinction between primary vascular disease and glomerulonephritis may be quite as baffling. An Italian, aged 55, was

admitted to the hospital with a typical acute glomerulonephritis following a streptococcus sore throat. The disease passed through a nephrotic stage of some duration after which the edema and proteinuria subsided and the blood pressure rose. All symptoms disappeared. The patient was seen at intervals thereafter for 17 years during which he had two elective operations. His heart became slightly enlarged but remained competent. His urine never contained more than a faint trace of albumin, occasional hyaline casts, and white blood cells. His blood NPN remained normal. The diagnoses of benign hypertension or nephrosclerosis were made repeatedly, although there is every reason to believe that his condition was the direct consequence of nephritis. Another patient at the age of 18 had an acute glomerulonephritis from which an early fatal outcome was predicted by the master, Volhardt. Having fled the rigors of a Dutch winter to spend his few remaining days in the more genial climate of Egypt, the patient miraculously improved. He led an active life without limitations until the age of 50, although his blood pressure was always slightly elevated and his urine always contained a small amount of albumin, a few red blood cells, and occasional casts. He died two or three years later with cholelithiasis and common duct obstruction, having meanwhile suffered two coronary occlusions. His blood nonprotein nitrogen remained within normal limits until his terminal illness. Autopsy revealed a characteristic picture of chronic glomerular nephritis. Both of these patients on the basis of symptoms and signs would have been placed in the class of arterial disease had it not been for their stories of earlier overt acute glomerulonephritis. If the disease had begun, as it frequently does, in a more subtle way, and if there had not been frequent examinations during its course in each case to establish its continuity, it would have been difficult, if not impossible, to ascertain the true nature of these maladies.

It is not uncommon to read in a case record a statement to the effect that hypertension must be of primary vascular rather than renal origin because the usual tests reveal no evidence of impairment of the function of the kidneys. These can only be regarded as careless remarks since the authors probably know that the loss of one kidney, provided the other is sound, has no effect on the function of the kidneys that is appreciable by available tests. In fact, renal insufficiency will not become obvious until one-quarter to one-half of the second kidney has been pared away. The statement in the record should more properly read: probably more than one-fourth of this patient's original kidney substance is intact, since his responses to functional tests are normal.

One of the reactions by which compensation for loss of kidney substance is achieved is hypertrophy of the remaining renal tissue. The kidneys have not the power to regenerate nephrons, but those that remain enlarge. Examination of the size of kidneys is a functional test that is too often neglected. Medical records contain many fatal tragedies when one kidney was removed under the impression that it was useless, without recognition that the presumably sound kidney was not larger, sometimes even smaller, than normal.

Whatever the mechanism of renal hypertension may be, it is not directly related to impairment of the function of the kidneys, but to restriction (not abolition) of the circulation to a certain proportion of the functioning kidney substance. In otherwise normal humans and dogs this proportion appears to be more than 50 per cent. The fact that unilateral injury apparently sometimes provokes hypertension which is relieved by removal of the injured kidney does not warrant generalization. If to produce hypertension, restriction of the circulation to more than one-half of the kidney substance is usually required, the appearance of hypertension with an apparently unilateral lesion may signify that the other kidney is not intact. In some cases in which hypertension has subsided after unilateral nephrectomy there is reason to believe that this was the case, since the blood NPN became permanently elevated after the operation. The lack of correlation between renal insufficiency and hypertension has been illustrated in many ways, of which intra-arterial injection of trypsin is one of the most gruesome and definitive. Injection of appropriate amounts of this material into one renal artery destroys the injected kidney without affecting the opposite organ. In the dog this induces no hypertension nor over-all renal insufficiency, though it does greatly impair the function of the injected kidney. If now a cuff is placed on the artery of the injected kidney, it has no obvious effect. If it is placed on the artery of the sound kidney, the blood pressure rises, but renal function is not disturbed. If, at this point, the injured kidney is removed, hypertension persists, but renal function is preserved. If, however, the sound kidney with constricted artery is removed, the blood pressure falls to normal, but the animal dies of renal insufficiency.

Removal of two-thirds or more of the renal mass produces insufficiency of the kidneys. This manifests itself first, not in increase of the blood NPN or other evidences of retention of waste products, but in polyuria. Impairment of excretory function, i.e., glomerular filtration, can be demonstrated by modern clearance techniques, but this does not prevent the elimination, in the course of the day, of the necessary amount of nitrogenous waste. Polyuria is mentioned as the first symptom of renal insufficiency because it

is the most evident fact, evincing itself in obligatory nocturia, diurnal frequency, and increased thirst. It has been rather generally held that the polyuria is a result of the inability to elaborate a concentrated urine. So far as failure to concentrate urine is a natural corollary of polyuria, this is a correct statement of fact; but it does not necessarily follow that the mechanism for reabsorption of water is specifically impaired. It has been demonstrated that the animal with insufficiency of the kidneys will not concentrate urine to the usual extent in response to dehydration or pitressin. Since polyuria is the first symptom of renal insufficiency, the concentration test should be the most delicate criterion of this condition. If conducted with care and consideration, it is extremely valuable; but as it is generally applied it is as likely to be misleading as helpful. It is the usual practice to measure the specific gravity of specimens of urine after obvious fluids have been withheld or restricted for 12 to 24 hours. Under these conditions a normal subject leading a usual life will yield a specific gravity of 1.028 or higher, since there is in his body no large excess of water, but an adequate amount of solutes requiring excretion, particularly urea and sodium chloride which contribute most to the solids of the urine. Patients, on the other hand, may be overhydrated, undernourished, or receiving inadequate diets. The overhydrated subject may not reach a great enough state of dehydration to pass a concentrated urine after only 12 to 24 hours of water restriction. In the presence of edema the test is useless because part of the edema fluid may be excreted in a urine with little salt. To subject a patient on a salt-poor diet to a concentration test is absurd. The malnourished subject may not concentrate because his nitrogen metabolism is greatly reduced or because he is storing so much nitrogen as protein that he has little to waste in the urine. Concentration is also impaired in diabetes insipidus and in profound anemia. Failure to concentrate under any of the conditions that have been mentioned cannot be attributed to renal insufficiency. A high urinary specific gravity when the urine does not contain peculiar solutes such as sugars, cystine, or diodrast, is strong evidence against renal insufficiency; failure to concentrate under the stress of the usual procedures is a far less reliable evidence for renal insufficiency. In the presence of nitrogen retention the concentration test is contraindicated even if it is not supererogatory.

The polyuria of renal insufficiency is the earliest and simplest sign that the conservative powers of the kidney have broken down. As reabsorption of water becomes inadequate, reabsorption of sodium chloride becomes impaired and gradually most of the other reabsorptive and secretory activities of the kidney tubules by which the normal composition of the internal

environment is maintained, among them the ability to excrete ammonia and to vary pH of the urine, suffer. Too much attention has been paid to the excretory offices of the kidney to the neglect of its conservative services. There is not time to discuss these in detail. Disorders of the kidney in disease must, however, be examined in the frame of modern renal physiology.

Renal insufficiency of even an advanced grade may be unaccompanied by nitrogen retention; but the subject with insufficient kidneys is more prone than the normal individual to develop nitrogen retention which may ultimately become an irreparable feature of the condition. There is no reason to believe that high blood urea or NPN is harmful; it can be regarded as an effective means to increase the excretion of nitrogenous waste products in the face of an inadequate filtering mechanism. It does, however, serve as a useful symbol or signal. One of Volhardt's most valuable contributions was the distinction between renal insufficiency and renal decompensation. As the criterion of insufficiency he chose the failure to concentrate urine, because it occurs when the quantity of actively functioning renal tissue is reduced below a necessary minimum; as the criterion of decompensation he selected elevation of blood urea or NPN. Kidneys that are actually or potentially normal may fail to excrete sufficient urea to keep the NPN from rising: if the quantity of urea offered for excretion is too great, if the fluid available for its excretion is too small, if the circulation of the kidneys is inadequate, or if there is any combination of these disadvantageous conditions. Patients with extreme dehydration and shock from gastro-intestinal disorders may have blood NPN's as high as any that may be encountered in the terminal stages of renal disease, but these will drop to normal as soon as adequate hydration and salt repletion are established. A patient with pneumonia or other disease that accelerates destruction of protein, given insufficient fluids, will have nitrogen retention. Cardiac failure impairs the excretory functions of the kidneys; shock has a more profound effect, even if it does not precipitate acute renal failure—lower nephron nephrosis. These are examples of renal decompensation without renal insufficiency, states in which the kidneys, though possessed of normal faculties, cannot exercise them to the best effect because they are working at a disadvantage. Decompensation must be distinguished from true renal disease with insufficiency so that its causes may be discovered and corrected. These same extrarenal disorders are operative in patients with renal insufficiency. It is of the utmost importance to ascertain the relative effects of each. Little can be done for renal insufficiency because destroyed renal substance cannot be

replaced; but it may be possible to correct renal decompensation, thus enabling the remaining renal substance to work more efficiently.

The term *uremia* and the concept it embodies imply the accumulation in the blood of products that belong in the urine. Though retention products may be the ultimate cause of death in anuria, it is doubtful whether any specific poisons are responsible for the ultimate dissolution. The multitudinous symptoms and signs covered by the term uremia in renal disease have little in common with those of anuria which, aside from the emotional stress, especially to the observers, are relatively undramatic. A large proportion of the phenomena termed uremic are, as Volhardt and Foster pointed out almost 40 years ago, vascular in origin, consequences of hypertension, arterial disease, and heart failure. Others are results, not of retention of waste products, but of failure of the conservative functions of the kidneys. If their true nature were more frequently analyzed in detail and corrective measures instituted for each, therapy would be greatly advanced. The term uremia has a defeatist ring that fosters complacency or routine procedures rather than thoughtful action.

I shall close with a description of the natural history of glomerular nephritis arising from streptococcal infections. It is highly probable that there are other types of nephritis. In the Civil War, the Franco-Prussian War, and the First World War, nephritis assumed epidemic proportions in certain areas. In 1918, for example, in a 1,000-patient Base Hospital in France, receiving from the Somme front, more than 200 cases of trench nephritis were seen within six months. Among these were frequently repeated syndromes of a bizarre nature, of which similar sporadic instances have subsequently been encountered in civil life that differed strikingly from the conventional picture of glomerular nephritis arising from streptococcal infections. Unfortunately, neither the British nor the American Military Forces were willing to undertake or even to facilitate an investigation of the condition because they felt that it was not a major cause of morbidity or mortality.

Scarlatinal nephritis will be selected as the central text because it has such a venerable history, but reference will be made to other streptococcal infections for comparison. The usual story is that the patient, having recovered from scarlet fever, two to four weeks after its onset develops hematuria, albuminuria, edema, hypertension, or any one or more of these disorders. This appears to be a somewhat inaccurate description of the sequence. If the patient is carefully scrutinized at the onset of the nephritis, evidences of a deep-seated streptococcal infection can be found: peritonsillar abscess, cervical lymphadenitis, otitis media, unresolved pneumonia, em-

pyema, etc. The sequence suggests that the subject, having become sensitized by the initial infection, responds with a nephritic reaction if infection by the same organism persists or recurs at the proper interval. Presumably this is not a reaction to the toxin because it occurs at a time when there is an excess of antitoxin in circulation. Lyttle found that two to four weeks after the onset of scarlet fever the Addis counts increased sharply in the urine of the majority of patients. Wyckoff detected a similar reaction after streptococcal sore throats without rash. These observations suggest that streptococcus infections provoke some reaction in the kidneys which, if infection persists when they occur, are exaggerated to a glomerular nephritis. Certain streptococci appear to be more prone than others to provoke such reactions and certain individuals appear to be more susceptible than others to these reactions. Oddly, males are more susceptible than females. In most series of acute nephritics the ratio of males to females varies from 2 to 3:1.

The incidence of nephritis as a result of streptococcal infections is hard to estimate because of the long interval between the initial disease and this sequel and because the acute manifestations may be so trivial. Reports of carefully observed epidemics of scarlet fever in institutions indicate that it may vary from 0.1 to 10 or even 20 per cent. This relatively low incidence makes a study of the epidemiology of acute nephritis in the general population difficult.

The great majority, 90 per cent or more, of patients who incur the disease recover completely. It follows that the characteristic lesions of the disease in its acute phase must be completely reparable, not the deeply destructive or heavily productive lesions that were described in the pathological textbooks of the past.

The outcome of acute nephritis bears little relation to the severity of its onset. The attack that begins with extreme hypertension, oliguria, heart failure, massive edema, and convulsions may subside completely and quite rapidly; one that opens with only microscopic hematuria and equivocal hypertension may progress to a fatal termination in a few months. This appears to be an adequate explanation of the fact that the origin of such a large proportion of cases of glomerular nephritis is obscure. Longcope, Ellis, and others have proposed that there are two types of nephritis: one that follows the pattern that has been described, the other cryptogenic. The strongest argument against this view is that the onset of a cryptogenic nephritis has not been observed. When glomerular nephritis begins in a patient under observation, its onset follows the characteristic pattern. Since cryptogenic nephritis (in the sense that a distinct origin cannot be traced)

makes up a large proportion of the clinical nephritis that is seen, it should surely occur occasionally in the patient population of a large hospital. There is some evidence to suggest that the duration and outcome of glomerular nephritis may depend on the persistence of the etiological agency, but this has not been clearly established.

Complete recovery from an attack of acute streptococcal glomerulonephritis appears to confer upon an individual an immunity to further nephritic reactions. A second attack of authentic glomerulonephritis is extremely rare. The individual does not develop any immunity to streptococcal infections, but only to the nephritic reaction. For example, one patient in our series with a peritonsillar abscess, preceded at the proper interval by a streptococcal sore throat, had a sharp, but short, attack of nephritis with gross hematuria, edema, and hypertension. Six weeks after the onset of this attack, when all signs of the disease had disappeared, he had another streptococcal sore throat followed at the proper interval by another peritonsillar abscess, but no hypertension or urinary abnormality. The frequent repetition of such sequences in large series of cases like Lyttle's, Seegal's, Loeb's, Longcope's, and our own, take on added significance when contrasted with the fact that if a nephritis does not clear up, subsequent streptococcal infections usually provoke exacerbations and progression of the disease. It is possible, of course, that the failure to react to the second infection is due to the fact that the second streptococcal agent was less nephrotoxic than the first; but second attacks of acute nephritis are so rare that this explanation seems hardly adequate.

There is not time to trace the variegated progress of the disease, but one of the steps in this progress deserves comment. A proportion of cases passes into a state termed *nephrotic,* characterized by edema, profuse proteinuria, hypo-albuminemia, and hyperlipemia, but without hypertension, hematuria, or evidences of renal insufficiency. Some of these patients recover completely as a larger number recover from the acute stage of the disease, proving that the essential lesion of the nephrotic syndrome is also reparable. The frequency with which this syndrome occurs in the course of glomerulonephritis, especially in children, identifies it as a stage in this disease. Because the syndrome frequently appears without recognizable nephritic antecedents, however, many have concluded that there is a separate entity, "idiopathic" or "lipoid" nephrosis. (In this connection amyloid disease and nephrotic syndromes arising from infiltrations of the kidney of various kinds are excluded from consideration.) The course of the cryptogenic disease cannot be distinguished from that of the nephrotic stage of glomerular nephritis. The strongest argument against the concept of idio-

pathic nephrosis is the one used against the concept of cryptogenic nephritis. Though the nephrotic syndrome is not extremely uncommon, especially in children, when the disease has been seen at its inception, it has invariably originated from glomerular nephritis.

Its natural history seems to identify glomerular nephritis with the reactive or, in a broad sense, allergic disorders. The pattern of the reaction is peculiar and quite specific. Its delineation has been the work chiefly of John Lyttle, the Loebs, the Seegals, and Longcope. We hope we have contributed our mite. That a typical glomerulonephritic syndrome can be produced in some persons by foods and other antigens has been demonstrated. There are, in our records, a few such cases. Nephrogenic nephritis, the result of a sensitization reaction, though it is initiated by administration of antigen for only a short time, progresses thereafter to a fatal termination as may glomerular nephritis.

To describe the contrast between the glomerulonephritic reaction and that of rheumatic fever—another probable reaction to streptococci—would require a separate lecture. The Seegals, Loebs, and their associates have published an excellent comparison. In spite of their differences the two reactions are not mutually exclusive. There is evidence that the frequency of both hypertension and glomerulonephritis is unduly large in patients with rheumatic fever. At least caution should be exercised in attributing albuminuria and hematuria in this disease to fever and heart failure, as is frequently done.

If recovery from acute glomerulonephritis confers an immunity to the nephritic reaction, it should protect persons against other disorders that arise from a similar reaction. The life history of patients who have recovered from acute nephritis should be highly informative. For example, toxemia of pregnancy is in its manifestations highly analogous to acute nephritis. If a patient has active nephritis, pregnancy will provoke an exacerbation of the condition. Will complete recovery from acute nephritis protect women from pregnancy toxemias? To obtain the answer to this question will require the careful accumulation of an enormous amount of material, particularly because the weaker sex is less susceptible to glomerulonephritis. From a small amount of material of my own collected over 25 years it seems to be possible to say that the woman who has recovered from acute nephritis has no enhanced susceptibility to toxemias of pregnancy. One of my patients at the last report had had seven pregnancies without complications and blood pressure and urine were still normal.

I should like to state again that this was not intended as a comprehensive discussion of renal disorders, but of certain points of view that seem to me

of value in approaching the analysis of these disorders. Emphasis has naturally been placed on those general aspects of these disorders that appear to me to be given too little weight in this era of direct measurements. Such measurements are of the greatest value for the control of therapy and for prognosis, but should be used with thoughtful discrimination. I wish to reiterate that streptococcal nephritis is probably not the only kind of acute nephritis. It was discussed at length as an example of an approach to the study of disease which might throw light on the nature of some of the other renal and vascular disorders and their relations to one another.

Because this lecture was intended less as an exposition of facts than a brief presentation of an approach to the discovery of fact, it has been impossible to qualify or document all the statements. For the same reasons no bibliography is appended.

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STUDIES ON INFLUENZA VIRUS RECEPTOR SUBSTANCE AND RECEPTOR-SUBSTANCE ANALOGUES

II. Isolation and Purification of a Mucoprotein Receptor Substance from Human Erythrocyte Stroma Treated with Pentane†

Agglutination of red cells by influenza virus, and the concept of "receptor" areas on the cell surface which are enzymatically modified after contact with virus, were described by Hirst in 1942.[17] Since that time much indirect evidence has been collected suggesting that virus receptors consist, at least in part, of mucoid substances; examples of this evidence are the inactivation of receptors by periodate[18] and the active competition with receptors by mucoids such as the "Francis inhibitor" of serum and ovarian cyst fluids,[8, 22, 23] ovomucin,[14, 21] and highly purified sheep salivary mucoid.[26]

Direct isolation of specific receptor-substance mucoids from the complex lipoprotein of the erythrocyte surface is obviously more difficult than the purification of receptor analogues from soluble mucoid secretions. Virus-inhibitory extracts have, however, been prepared from red cells of various species by boiling stroma in water[15] or by extracting red cells in a Waring blendor.[13] A purified preparation isolated from human red cell stroma in the first instance as an ether-soluble lipomucoid was described in 1948 by deBurgh, Yu, Howe, and Bovarnick.[5] Their most active fraction contained at least 50% reducing substances but was said to contain neither hexosamine nor phosphorus. In the same year Woolley[41] obtained a heat-labile polysaccharide substance from the supernatant fluid of hemolyzed erythrocytes which was shown to antagonize the antihemagglutinin effect of apple pectin. After exposure to influenza virus the purified erythrocyte substance was no longer capable of inhibiting pectin, and it was concluded that the former material, which itself showed detectable inhibition of PR8 virus at 0.04 μg/ml.,[42] was the substrate for enzymic attack by virus on the red cell surface. In an extensive study by Howe,[19] which appeared while the present work was in progress, physical methods were described for the isolation

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by high-speed centrifugation at controlled pH and ionic strength, of stroma fractions rich in virus inhibitor and blood-group substance. In the majority of these cases, however, detailed chemical investigations of the various preparations were not described.

Although we have found the method of deBurgh *et al.* relatively convenient to carry out in the laboratory, the yield of active material is extremely low, and, even after further purification, the most active fraction is markedly inhomogeneous in the ultracentrifuge.[25] We have also prepared an active virus-inhibitory lipomucoid by extracting dried red cells with dilute ethanol, as originally described by Hallauer[18] for the isolation of blood-group substance, but the method is cumbersome and the yield is again extremely small. It has seemed desirable, therefore, to investigate alternative methods in an attempt to improve the yield and, if possible, the homogeneity, of extracted receptor-substances.

In the present investigation, human red-cell stroma were treated with pentane in the hope that active material might be released into the solvent. Neither virus-inhibitor nor blood-group substance were, however, present in the pentane extract, but it was observed that after treatment with this solvent at room temperature for several days the stroma were converted into a stable amorphous gel. It seemed likely that stroma in this finely divided state would be suitable for further extraction, and it was subsequently found that relatively large amounts of active material passed into solution when the stroma gel was extracted with 50% ethyl alcohol. No active material was obtained if stroma were extracted without the preliminary pentane treatment.

This paper describes some of the properties of a virus-inhibitory mucoprotein obtained in relatively high yield from human group A stroma treated by the pentane-dilute alcohol process. The mucoprotein has been examined chemically and in the ultracentrifuge and electrophoresis apparatus. The inhibitory activity of the various mucoprotein fractions has been estimated against several active and indicator strains of influenza virus, and the destruction of inhibitory activity confirmed on exposing purified receptor-substance to active influenza virus or the receptor enzyme of *V. cholerae.*

MATERIALS AND METHODS

Virus. "Reference" strains of influenza A and B virus, in the form of infected allantoic fluid, were obtained through the kindness of Doctor Alick Isaacs of the World Influenza Centre, Mill Hill, London. "Indicator" forms of strains LEE, MEL, and PR8 (i.e., H.LEE, H.MEL, and H.PR8) were prepared by heating the allantoic

fluids at 37° or 55° C. under the conditions described by Stone.[38] Virus in this form is considerably more sensitive to inhibition by mucoids than active (unheated) virus.[12]

Receptor-destroyer enzyme. A purified preparation of *V. cholerae* receptor-destroying enzyme was kindly provided by Sir Macfarlane Burnet. The material had been purified as described by Ada and French.[1]

Titration of viral antihemagglutinin. The method employed throughout these studies was essentially that described by Anderson.[2] In summary, a series of double dilutions of the extracts was prepared in 0.2 ml. of normal saline; ten agglutinating units of virus in the same volume of saline were then added to each, and the mixture held at 4° C. for one hour. Finally, 0.2 ml. of a freshly prepared 1% suspension of pigeon or "sensitive" fowl erythrocytes were added, and the pattern of agglutination read 1 to 1½ hours later. In the majority of cases pigeon cells were used since they possess the added advantages of rapid settling and high stability as well as giving titres of the same order as the most sensitive fowl cells. The titres obtained with the pigeon erythrocytes from our stock did not vary significantly from bird to bird.

Particular care was taken to standardize the titrations of inhibitory activity as far as possible, since the subsequent procedures were to a large extent conditioned by them. A "standard" preparation was included in all tests, and the titrations of any one series of fractions were carried out at the same time with the same virus suspension. The latter was titrated immediately before use, diluted to give ten agglutinating doses per unit volume, and the titre of the dilute preparation checked against the freshly prepared red-cell suspension. The relatively powerful dose of ten units was used throughout because it was observed in earlier experiments with red-cell extracts that there is not necessarily an inverse relation between virus dose and inhibitory titre over the lower range of virus concentration. With a low virus dose, e.g. two agglutinating units, partial inhibition may be observed to a relatively high titre and a sharp end-point is difficult to obtain. A similar phenomenon has been observed by Doctor C. M. Chu (personal communication 1950) with "normal" inhibitor in mouse sera against certain influenza virus strains.

Titration of blood-group activity. These titrations were carried out with standard human anti-A sera according to the usual agglutination-inhibition technique.[30]

Analytical methods. Nitrogen was determined by the micro-Kjeldahl method using the apparatus of Markham; P by the method of Briggs[4] reducing sugar by the method of Somogyi,[30] N-acetyl-hexosamine and hexosamine by a modification of the methods described by Morgan and Elson[29] and Elson and Morgan,[11] respectively, and methyl pentose by the method of Dische and Shettles[10] using a "Uvispek" spectrophotometer.

Paper chromatography for amino acids was carried out according to the methods described by Consden, Gordon, and Martin[9] and for sugars and hexosamines as described by Partridge.[32] The aniline hydrogen phthalate reagent[33] was used for identifying sugars. All chromatograms were run on Whatman No. 4 paper, the solvents being s-collidine, butanol-acetic acid, and phenol-ammonia.

Stroma. Stroma were prepared from washed human group A red cells by precipitation in the cold at pH 5.6 as described by Jorpes.[20] Packed stroma, prepared in batches of approximately 200 ml., were stored at −10° C. until 1-2 liters had been accumulated. They were then thawed, pooled, and treated as described in the following sections.

EXPERIMENTAL

Preparation A 1. Stromata (1 liter) was added to 2 liters of pentane with vigorous stirring. The emulsion was shaken for several hours daily for four weeks and then passed through a Sharples Supercentrifuge; the supernatant fluid, which contained neither virus inhibitor nor blood-group substance, was discarded. The deposited stroma gel was collected and resuspended in 2 liters of pentane, shaken for a further ten days, and again passed through the Supercentrifuge. The stroma gel, which still contained a considerable amount of pentane, was suspended in 2.5 liters of 50% alcohol. After shaking for several hours, a finely divided stable colloidal dispersion was formed, which, after standing for 14 days, was passed four times through the Sharples centrifuge. The clear, orange-colored supernatant fluid (extract A 1-1) was concentrated to small volume *in vacuo* and stored at –10° C. The stroma deposit, resuspended in 2 liters of 50% alcohol, was again extracted for seven days and then passed through the centrifuge as before (extract A 1-2). The concentrated supernatant fluids were freeze-dried, extracted with absolute alcohol in a Soxhlet apparatus, and washed with ethanol, alcohol-ether, and acetone: yield, 33.3 gm. from extract A 1-1 and 4.2 gm. from extract A 1-2. The stroma residue was again extracted with 10% alcohol for several days, but no further active material was recoverable.

Preparation A 2. Pooled stromata (2 liters) was shaken for three weeks with 9 liters of pentane and the gel-like mass extracted successively with 15 liters (extract A 2-1) and 12 liters (extract A 2-2) of 50% alcohol over a period of several weeks. The supernatant fluids were concentrated *in vacuo* to approximately 300 ml. and centrifuged on the "Servall" angle centrifuge at 8,000 r.p.m. to remove any finely divided insoluble particles. Active virus-inhibitory material was precipitated from the slightly opalescent supernatant fluids as described below.

ETHANOL FRACTIONATION OF THE EXTRACTS

Preparation A 1. The first freeze-dried extract (A 1-1) was taken up in 400 ml. of water, stirred for one hour, and centrifuged. The active material was precipitated from the supernatant fluid by the addition of 900 ml. of ethanol in the presence of potassium acetate. After dialysis and freeze-drying 3.3 gm. of brown powder were obtained. A second fraction, collected by increasing the ethanol concentration to 80%, consisted of dark

brown tarry inactive material. The second extract (A 1-2) was fractionated with ethanol at the same level, yielding 0.7 gm. of dark brown powder.

Preparation A 2. Ethanol (1 liter) was added with stirring in the presence of potassium acetate to 700 ml. of the pooled concentrated extract A 2-1. The heavy flocculent precipitate was collected on the centrifuge, washed twice with 90% ethanol, and dried *in vacuo*—weight 43 gm. Extract A 2-2 was similarly treated with 1.8 volumes of ethanol and yielded 3.2 gm. of dark brown material. All the antihemagglutinin activity was recovered in these precipitates.

TABLE 1. ANALYSIS AND VIRUS INHIBITORY ACTIVITY OF FOUR STROMA FRACTIONS PRECIPITATED WITH ETHANOL

	Analysis			*Inhibitory activity*	
Material	*N%*	*P%*	*Hexosamine %**	*H.LEE†*	*H.MEL‡*
Fraction A1-1	9.1	1.5	10.0	1200	1280
Fraction A1-2	10.1	1.5	8.8	2000	2560
Fraction A2-1	9.6	1.0	10.0	1000	1280
Fraction A2-2	10.5	0.7	10.6	1200	1800

* Calculated as glucosamine-HCl.
† Titre of 1% solution against 10 units of indicator LEE virus.
‡ Titre of 1% solution against 10 units of indicator MEL virus.

Analytical figures for the four alcohol-precipitated fractions are given in Table 1, together with inhibitory activities against two indicator virus strains. It will be seen that the nitrogen content of the four preparations is reasonably uniform, varying from 9.1 in A 1-1 to 10.5 in A 2-2. All contain both hexosamine and phosphorus in contrast to the findings of deBurgh *et al.*[5] for ether-extracted receptor-substance. In our hands, however, preparations made by the ether-extraction process at a comparable stage of purity contained 3.4% N, 5.6% hexosamine, and 1.3% P.[27] Active fractions isolated by differential centrifugation contained 8-13% N and 1-1.5% P.[19] All four preparations inhibit hemagglutination by indicator LEE and MEL viruses to equivalent titres, the second of each pair of fractions tending to be more active than the first against both virus strains. As has been observed with most partially purified erythrocyte extracts, indicator MEL virus is inhibited to higher titre than indicator LEE, although in this case the difference is relatively small.

As the various fractions were similar in composition and activity, the three main ones (A 1-1, A 1-2, and A 2-1) were mixed, resuspended in 750 ml. of 50% ethanol, shaken at intervals for ten days, centrifuged, and the insoluble residue again extracted with 50% ethanol three times. The alcoholic extracts were pooled, centrifuged at 8,000 r.p.m. for 30 minutes, and the clear, straw-colored supernatant fluids concentrated *in vacuo* and dried from the frozen state. The material (6.8 gm.) was completely dissolved in 70 ml. of distilled water, and two volumes of ethanol were added cautiously at 0° C., a trial fractionation on a small aliquot having shown that the active component precipitated uniformly between 1.25 and 1.75 volumes of ethanol. The flocculent precipitate was collected on the centrifuge, redissolved in water, dialyzed against several changes of distilled water for 48 hours at O° C., and finally freeze-dried. The yield was 2.8 gm. of very pale yellow material (AP-1).

At this stage the titre of a 1% solution of the partially purified muco-protein was 4,800 against H.LEE and 3,500 against H.MEL. Considerable loss of activity had, therefore, occurred during the later stages of the purification process, since the weight of active material had been reduced from 41 gm. to 2.8 gm. with only an approximately two-fold increase in activity. Further, antihemagglutinin titre against H.MEL was now slightly lower than against H.LEE. Purification was continued, however, since the rigorous chemical treatment was designed to obtain a homogeneous preparation rather than one of exceptional inhibitory activity.

Treatment with acid alcohol-ether. A portion (2 gm.) of fraction AP-1 was dissolved in 40 ml. of distilled water and added drop by drop to 200 ml. of 60-40 alcohol-ether containing 0.5% hydrochloric acid and maintained at –40° C.[28] Stirring was continued for four hours, after which time the insoluble material was removed by centrifugation at 0° C. and redissolved in 30 ml. of cold distilled water. The extraction process was then twice repeated. Finally, the extracted material was dialyzed against several changes of distilled water at 4° C., spun in the angle centrifuge at 8,000 r.p.m. for 30 minutes, and freeze-dried. The final product (AP-2) weighed 1.6 gm. The virus-inhibitory activity was not reduced by this treatment.

Treatment with the Sevag technique. A portion (0.5 gm.) of fraction AP-2 was exhaustively treated by a modified Sevag deproteinization process. The material was dissolved in 50 ml. of 0.2 M acetate buffer at pH 5.7 and stirred vigorously with 20 ml. of chloroform-butanol for one hour. The white colloidal deposit of denatured protein was removed by centrifugation, and the process continued until no further protein came out

of solution. Approximately 15 treatments were required. The supernatant fluid was then dialyzed free from buffer at 4° C. and freeze-dried, yielding 120 mg. of pure white material which dissolved rapidly to give a colorless, faintly opalescent solution. The denatured protein was shaken for several days in a large volume of buffer, centrifuged, and the supernatant fluid dialyzed and freeze-dried; 102 mg. of whitish powder was so obtained. A second 0.5 gm. portion of AP-2 was treated in the same way with the exception that the pH of the acetate buffer was 6.7 instead of 5.7.

TABLE 2. ANALYSIS OF ERYTHROCYTE MUCOPROTEINS AFTER TREATMENT WITH THE MILES-PIRIE AND SEVAG TECHNIQUES

Fraction	*N%*	*P%*	*S%**	*Reducing sugar†*	*Hexosamine‡*
Pool—AP-2	10.0	0.46	2.6	14.7	9.5
Sevag treatment	5.3	–	–	13.7	6.3
Sevag treatment residue	7.7	–	–	11.0	7.3

* Determined by Drs. Weiler and Strauss, Oxford.
† Hydrolysed with 0.5 N HCl for 24 hours; calculated as glucose.
‡ Hydrolysed as above; calculated as glucosamine-HCl.
– Not determined.

CHEMICAL PROPERTIES OF THE ISOLATED MUCOPROTEINS

All the fractions obtained in these experiments were readily and completely soluble in water. Before Sevag treatment, fraction AP-2 became faintly opalescent on the addition of an equal volume of 10% trichloracetic or sulphosalicylic acids. There was no precipitation on boiling or on adding picric or phosphotungstic acids, but slight precipitation occurred with 5% tannic acid and there was heavy precipitation with basic lead acetate. The biuret and ninhydrin tests were positive. After Sevag treatment the opalescence on adding trichloracetic and sulphosalicylic acids was very faint, but the other properties remained unaltered.

The analytical figures for the products obtained after extraction with acid alcohol-ether (AP-2), and after treatment by the Sevag technique, are given in Table 2.

Comparison with the analytical figures obtained for the earlier fractions (Table 1) shows that, as would be expected, the alcohol-ether treatment significantly reduced the P content without altering the other figures. The hexosamine: N ratio remained approximately 1:1. After the Sevag treat-

ment at pH 5.7, however, the N content was reduced by almost 50% but the hexosamine : N ratio was only slightly increased at 1 : 1.2.

As described in a later section, an active (cathode) fraction was separated from the Sevag-treated material by electrophoresis; the analytical figures for this fraction were N, 6.3%, reducing sugar, 15.3%, and hexosamine, 7.4%. The hexosamine : N ratio of 1 : 1.18 was, therefore, not significantly different from that of the Sevag-treated material before being submitted to electrophoresis.

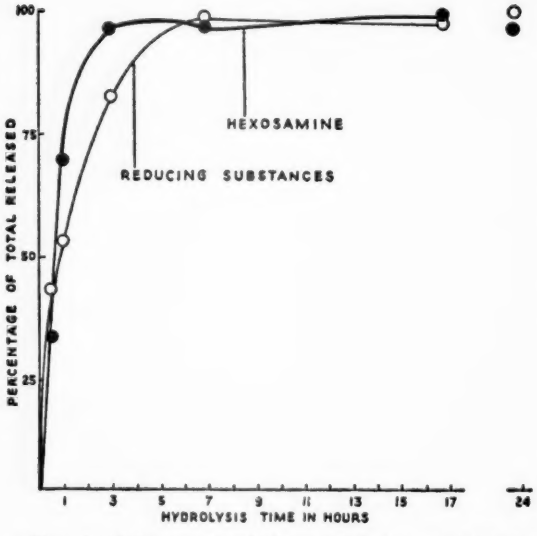

FIG. 1. Release of reducing substances and hexosamine from erythrocyte fraction AP-2 heated at 100° C. with 0.5N HCl.

A small amount of firmly bound lipid remained even after repeated acid alcohol-ether extraction. This could be demonstrated by hydrolyzing the extract with 0.5N hydrochloric acid or N acetic acid for eight hours and then extracting the hydrolysate several times with three volumes of ether and pentane. After evaporation, a small amount of fatty residue, equivalent to 4% of the original material, was recovered from the organic solvents. The nature of this lipid has not been investigated further.

Hydrolysis curve. The liberation of reducing substances and hexosamine during hydrolysis of fraction AP-2 was followed by sealing aliquots of a 1% solution in small glass ampoules with 0.5N hydrochloric acid, and immersing in a boiling water bath. Ampoules were removed at intervals and the contents carefully neutralized with sodium hydroxide. Typical results are illustrated in Figure 1. It will be seen that both reducing substances and hexosamine are released very rapidly under these conditions; the maximum value for reducing sugar is reached at 24 hours and for hexosamine at 17 hours. A considerable amount of charring takes place during hydrolysis with hydrochloric acid, and also, though to a lesser extent, with sulphuric or acetic acids.

Chromatographic examination of hydrolysate. The hydrolysis products were examined for reducing sugars, hexosamines, and amino acids by means of paper partition chromatography. In preliminary experiments considerable difficulty was encountered with severe "trailing," particularly when the papers were sprayed with the aniline hydrogen phthalate reagent for sugars. This difficulty was partly, though not entirely, overcome by the following technique: 10-20 mg. of fraction AP-2 were hydrolyzed overnight in 5N or 0.5N hydrochloric acid, and the hydrolysate then extracted twice with three volumes of ether and once with three volumes of pentane. "Trailing" was less marked when the more concentrated acid was used for hydrolysis. After centrifugation, the water layer was evaporated to dryness in a vacuum desiccator over sulphuric acid and caustic soda. The residue was taken up in one ml. of distilled water, approximately three mg. of Norite charcoal added, and the mixture brought to boiling point for one minute. After filtration the crystal-clear filtrate was again taken to dryness *in vacuo*. The residue, taken up in a small volume of water, was then applied to the paper strips and runs carried out in s-collidine, butanol-acetic acid, and phenol-ammonia with a minimum of "trailing." In one experiment the ether-extracted hydrolysate was passed through columns of the ion-exchange resins "Deacidite" and "Amberlite 1RC-50H" instead of being heated in the presence of charcoal, but the chromatographic results were inferior to those obtained after charcoal treatment.

In the final runs, two aliquots of hydrolysate were run on each paper together with "known" mixtures of galactose, glucose, glucosamine-hydrochloride, and chondrosamine-hydrochloride. The papers were then cut in two, one half being sprayed with the aniline hydrogen phthalate reagent, and the other half with the hexosamine reagents. Two pink spots with R_F values equal to those of the "known" glucosamine and chondrosamine hydrochlorides were obtained in collidine, and two similar but poorly separated spots in butanol-ammonia; a single spot only was obtained in phenol. The addition of glucosamine and galactosamine to the hydrolysate increased the intensity of the hexosamine spots, but no additional spots appeared.

In all solvents definite spots were found with R_F values corresponding to galactose and fucose, respectively. A further faint spot with the same R_F as glucose was also observed, but as the color was distinctly different, the identity of this spot is still doubtful.

Chromatograms were also run for amino-acids after hydrolysis in 6N hydrochloric acid. Ten spots were obtained on spraying with the ninhydrin reagent, corresponding to lysine, arginine, glycine, alanine, proline, valine,

leucine, and phenylalanine. Chondrosamine and glucosamine were also identifiable on these chromatograms.

Solubility in liquid phenol and diethylene glycol. The mucoprotein (fraction A 2-1) was completely soluble in 95% liquid phenol. A 1:1 mixture of liquid phenol and ethanol was cautiously added to the solution, but only approximately 10% of the material was precipitated when the ethanol concentration reached 50%. The activity of this precipitated fraction was slightly less than that of the original mucoprotein. Fractions precipitated above 50% ethanol concentration were largely insoluble in water. The exceptionally high solubility of the mucoprotein in liquid phenol is paralleled by the behavior of the sheep salivary mucoprotein.[29]

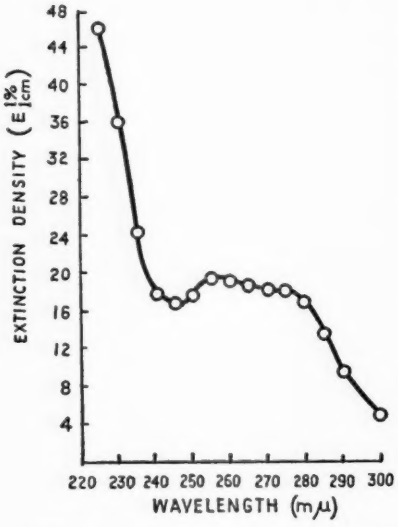

FIG. 2. Ultraviolet absorption spectrum of fraction AP-2 (0.04% aqueous solution).

The erythrocyte mucoprotein was also completely soluble in diethylene glycol, a solvent used extensively for the purification of bacterial mucoproteins. After high-speed centrifugation and passage through a Berkefeld candle, the clear solution was fractionated with ethanol at various levels between 30% and 80% final concentration. A small amount of material only was precipitated up to the 40% level, and fractions precipitated above this level were completely inactive. From these experiments it was clear that neither phenol nor diethylene glycol could be used in further purification of receptor-substance mucoproteins.

PHYSICAL PROPERTIES OF THE MUCOPROTEIN

Ultra-violet absorption spectrum. The ultra-violet absorption of fraction AP-2 was examined in the "Uvispek" spectrophotometer, using a one cm. cell and an 0.04% solution in distilled water. The curve (Fig. 2) shows a broad, ill-defined peak extending over the range 245-280 mμ. It is probable that the absorption at 245-260 mμ represents nucleoprotein; absorption from 270-280 mμ is probably due to aromatic amino acids.

*Sedimentation.** A 1% solution of extract AP-2 before and after Sevag treatment was examined in the ultracentrifuge at pH 8 in phosphate buffer (I = 0.1 + 0.15M NaCl) and at pH 4 in acetate buffer. The material sedimented very slowly at both pH 4 and 8, showing a fairly well-defined single symmetrical peak with little evidence of polydispersion. The peak appeared rather more sharply defined after Sevag treatment (Fig. 3).

*Electrophoresis.** One per cent solutions of fraction AP-2 before and after Sevag deproteinization were examined at pH 4 and 8 in the Tiselius apparatus. Before deproteinization, three broad, ill-defined components were

FIG. 3. Sedimentation diagram of 1% solutions of fraction AP-2; (a) before Sevag treatment; (b) after Sevag treatment. Phosphate buffer pH 8, I = 0.1 + 0.15M NaCl. Exposures taken 40 min. after reaching full speed, 60,000 r.p.m. (240,000 G).

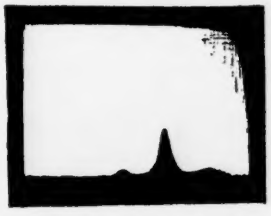

FIG. 4. Electrophoresis diagram of 1% solution of fraction AP-2 after Sevag treatment. Phosphate buffer pH 8, I = 0.2. Exposure taken after separation for 300 mAH.

observed. Two well-defined and widely separated peaks were observed after Sevag treatment (Fig. 4). It was possible to separate these peaks in the electrophoresis apparatus, and, as shown in the following section, activity was associated with the larger slower-moving component only.

BIOLOGICAL PROPERTIES OF THE EXTRACTED MUCOPROTEINS

Virus inhibition spectrum. The "inhibition spectrum" of various fractions against a range of indicator and active influenza virus strains is given in Table 3.

Fraction AP-2 (pooled fractions soluble in 50% ethanol, after extraction with acidified alcohol-ether), showed the highest virus-inhibitory activity of all fractions obtained in the present experiments. The titre of 10,000 against indicator LEE was comparable to that of purified serum mucoid fractions,[22] but considerably lower than the activity of purified mucoproteins isolated from sheep salivary glands.[20] The titre of fraction AP-2 against H.LEE actually increased after treatment with alcohol-ether, a phenomenon not yet

* These procedures were carried out through the kindness of Dr. R. A. Kekwick.

explained, but observed also with serum mucoid[22] and erythrocyte mucoproteins extracted in ether. A significant increase in inhibitory activity was observed against all indicator virus strains as compared to active (unheated) virus: in the case of LEE and indicator LEE an approximately 20-fold increase occurred with each fraction shown in the table. Another point to be noted is that, with these preparations, the titre against H.MEL was lower than that against H.LEE.

In contrast to the result expected from previous experience with receptor analogues, very extensive loss of inhibitory activity against all strains occurred after Sevag treatment at either pH 5.7 or 6.7: the titre against H.LEE, for example, was reduced to approximately 20% of the original

TABLE 3. ANTIHEMAGGLUTININ ACTIVITY OF 1% SOLUTIONS OF ERYTHROCYTE MUCOPROTEINS BEFORE AND AFTER SEVAG TREATMENT

Fraction	*LEE*	*H.LEE*	*MEL*	*H.MEL*	*PR8*	*H.PR8*
Pool—AP-2	500	10,000	500	4,000	800	3,200
Pool—Sevag treated pH 5.7	10	1,750	10	100	10	10
Pool—Sevag residue pH 5.7	500	9,000	100	4,000	700	2,800
Pool—Sevag treated pH 6.7	100	2,800	10	600	80	300
Pool—Sevag residue pH 6.7	180	9,600	10	2,800	750	2,400

figure, and the activity against other strains was decreased to an even greater extent. Thus, after treatment at pH 5.7, none of the active strains were demonstrably inhibited, nor was H.PR8. As found with other erythrocyte extracts, activity against active MEL and H.PR8 is particularly sensitive to destruction during chemical treatment, while that against H.LEE is the most stable. At pH 6.7 some activity remained against active LEE and PR8, and against H.PR8, but it is clear that even the relatively milder conditions have brought about severe loss of inhibitory activity. It should be noted, however, that the soluble mucoprotein extracted from the precipitated protein gel resulting from the Sevag treatment showed activity of similar order to that of the original material (before Sevag treatment) against strains LEE, H.LEE, H.MEL, PR8, and H.PR8, but again the titre against MEL was significantly reduced.

Effect of electrophoresis. The virus-inhibitory activity of the two electrophoretically separated components from the Sevag fraction treated at pH 5.7 is given in Table 4.

The results obtained for the electrophoretically separated components are of considerable interest. The small anode component (Fig. 4) was com-

pletely inactive toward all strains, but the larger, slower-moving cathode peak was associated with the slightly higher activity against H.LEE than was the starting material. It is clear, however, that complete concentration of activity against this strain has not been achieved, since the cathode component represents only 5% of the total material (100 mg.) subjected to electrophoresis.

Inactivation by influenza virus and RDE. Sufficient data may be given here to show that the inhibitory action of the purified erythrocyte extract is progressively lost on incubation with living influenza virus or the "receptor-destroying" enzyme of *V. cholerae*[37] as has previously been described for purified ovarian cyst mucoid[9] and purified sheep salivary mucoid.[39] More

TABLE 4. VIRUS INHIBITORY ACTIVITY OF ELECTROPHORETICALLY SEPARATED ERYTHROCYTE FRACTIONS

Fraction	*LEE*	*H.LEE*	*MEL*	*H.MEL*
Sevag treatment pH 5.7	<10	1,750	<10	100
Cathode component	<10	2,500	<10	<10
Anode component	<10	<10	<10	<10

detailed studies now in progress on the kinetics of inactivation with a series of virus strains will be described in a later paper.

Aliquots of a solution of purified erythrocyte extract (AP-2) were incubated with 100 agglutinating units of fresh LEE or MEL virus diluted in acetate buffer pH 6.4 containing 0.1% $CaCl_2$. Control tubes with heat-inactivated virus and without virus were included. After two hours' incubation at 37° C. the tubes were removed from the bath and brought to 100° C. for one minute to destroy the active virus. It was found that active LEE virus reduced antihemagglutinin activity against H.LEE to 30% and against H.MEL to less than 1% of the control values. Active MEL virus reduced the titres against the two strains by 50% and 70% respectively.

Aliquots of purified erythrocyte extract were also incubated with a purified eluate of *V. cholerae* receptor-destroying enzyme (RDE) diluted in calcium acetate buffer pH 6.4. After incubation at 37° C. for two hours with an equal volume of a 0.005% solution of RDE, a solution of erythrocyte extract showing originally a titre of 6,000 against H.LEE and 4,000 against H.MEL had become completely inactive (less than 1 : 10) against both strains.

Inactivation by periodic acid. Like the red cell receptor[18] and the soluble Francis inhibitor in normal serum and purified serum mucoid[22] the inhibi-

tory activity of the purified erythrocyte extract is sensitive to the periodate ion. Exposure of a 1% solution of fraction AP-1 in acetate buffer at pH 5.0 to 0.005M periodic acid for one hour at room temperature reduced the titre (H.LEE) from 9,600 to 10.

Action of trypsin. A 0.5% solution of purified fraction AP-2 was incubated for two hours with an equal volume of 2% "Difco" trypsin in borate buffer pH 8.1, controls containing heat-inactivated enzyme being included. No significant reduction in titre occurred against LEE, H.LEE, MEL, or H.MEL.

Blood-group activity. The anti-A and anti-H activity of the extracts was estimated at various stages during the fractionation process. No anti-A activity was detected in the crude extracts, and only minimal (less than 1 : 10) inhibition of anti-H sera. The purified fraction AP-1 was kindly examined in detail by Miss Joan Thompson, who reported no inhibition (less than 1 : 2) of anti-B, anti-M, or anti-D sera, and inhibition titres of 64 and 512 against anti-H and anti-A sera respectively.

DISCUSSION

The receptors of influenza virus exist on the erythrocyte surface presumably as part of a lipomucoid complex. The first stage of the chemical isolation of receptor-substance in methods so far described involves preliminary treatment with a concentrated lipid solvent which releases part of the complex in an extractable form; this is achieved in the process of deBurgh *et al.*[5] by heating (or freezing and thawing) stroma with ether, and with dried red cells by prolonged extraction with 95% alcohol.[16, 27] It is probable that in both these processes only a small fraction of the total receptor-substance is released from the stroma.[19] Further purification in all processes depends essentially on removal of firmly bound lipid from the extracted lipomucoid and fractionation of the protein components.

The chief advantages of the pentane-dilute alcohol process described in this paper are, first, the virtual absence of blood-group activity which is constantly associated with erythrocyte extracts prepared by the ether extraction of stroma or alcohol extraction of freeze-dried red cells[19, 27]; and, secondly, the isolation of a product which is completely and rapidly soluble in water and which retains constant activity even after storing in the dry state for up to two years. In our hands, erythrocyte extracts prepared by other methods frequently become partially insoluble after storage *in vacuo* and may also become largely insoluble after storing in solution at –10° C. for several days. This applies particularly to preparations made by the

ether-extraction process. The final yield of active substances with the pentane method is considerably greater than with the alcohol extraction of freeze-dried cells or with the ether extraction of stroma. It is clear, however, that none of the chemical methods so far described for preparing receptor-substance even approaches the ideal.

The presence of two hexosamines in the erythrocyte mucoprotein seems established on chromatographic evidence. Two spots with R_F values equal to those of glucosamine and galactosamine were invariably found, and no further spots appeared when the two known hexosamines were added to the hydrolyzate. However, definitely to establish the identity of the two hexosamines it would be necessary to isolate crystalline derivatives from large amounts of extract—a procedure which has not yet been possible. In this connection it may be noted that the single hexosamine present in sheep salivary mucoid was found to have the same R_F value as galactosamine in various solvents, but the melting point of the dinitrophenyl derivative was distinctly lower than that of the authentic galactosamine compound.[39] The presence of galactose seems reasonably certain; this sugar has been found in all erythrocyte extracts so far examined. It is probable that galactose occurs in small amounts only, since the spots obtained were weak, usually fainter than those of fucose. The presence of fucose, which is found in small amounts in all red cell extracts so far examined, was confirmed both by chromatography and the spectrophotographic method. It is of interest that the electrophoretically homogeneous mucoprotein of human urine,[40] which also actively inhibits influenza virus hemagglutination, contains glucosamine, galactosamine, galactose, mannose, and fucose.[14a]

The question of homogeneity of this, and in fact in all preparations of large molecules, is a difficult one; electrophoresis and ultracentrifugation provide some evidence of the range of variation in mobility and molecular size, but one is always faced with the possibility of dealing with mixtures of molecules of different biological specificity which show similar physical behavior in an electrical or sedimentation field. The present preparation shows a single peak even after prolonged sedimentation in the ultracentrifuge, little evidence of polydispersion, and extremely slow sedimentation, thus indicating the presence of small molecules of the same order of size: the molecular weight is probably less than 87,000 since the erythrocyte mucoprotein moves more slowly than the homogeneous mucoprotein isolated from sheep salivary glands.[26] The electrophoretic pattern before Sevag treatment is a complicated one with at least three differnt components; after Sevag treatment two sharp, well-separated peaks are obtained, inhibitory activity (against H.LEE) being associated with the considerably

larger slow-moving peak only. In this instance, therefore, an electrophoretically and ultracentrifugally homogeneous mucoprotein has been isolated from erythrocytes with moderate antihemagglutinin activity against one virus strain.

The extensive and unexpected loss of activity against all virus strains after mild Sevag treatment is difficult to interpret. In previous studies on the serum mucoid "Francis inhibitor" it had been found that antihemagglutinin activity against H.LEE was lost only if Sevag treatment was carried past a certain stage, but that activity against certain influenza A strains ("non-specific inhibitor") was destroyed very rapidly. These findings, together with other differences, were considered by McCrea[22] to establish the non-identity of the Francis and non-specific inhibitors, the former being associated with serum mucoid and the latter with the more labile serum proteins. This problem has also been discussed by other workers (e.g. Smith, Wilson, and Westwood[35]) who consider that it is more probable that the two inhibitors exist as different components of one complex. It is possible that Sevag treatment in the present instance has inactivated one part of a large molecule (that part inhibiting active and indicator A strains) while leaving the more stable component inhibiting H.LEE relatively intact. The experimental evidence obtained in the present investigation is against this interpretation, however, since quantitative recovery against the other strains (with the exception of MEL) was obtained from the deposited protein gel. Thus, it would appear that a *separation* of a nonprecipitated H.LEE inhibitory component has been obtained, and not simply inactivation of one component or group of components in a complex molecule. It will be noticed that the active material washed out from the protein gel resulting from Sevag treatment is similar in composition to the fraction remaining in solution (Table 2), the hexosamine : N ratio being practically unchanged. This suggests that one is dealing with a mixture of chemically very closely related mucoproteins, the chief distinction being that the inhibitor of H.LEE is less readily precipitated by chloroform.

The virus "inhibitor spectrum" of the pooled material, before Sevag treatment, is H.LEE > H.MEL > H.PR8 > PR8 > LEE=MEL. In the majority of erythrocyte extracts prepared by other methods, inhibitory titre against H.MEL is approximately twice as high as against H.LEE, the reversal in the present instance probably being a reflection of the rigorous chemical treatment. In the "crude" extract from pentane-treated stroma the titre against these two indicator strains is scarcely significantly different. It is perhaps impossible to compare the activities of erythrocyte extracts prepared by different methods, since the relative degree of purity is difficult

to assess; however, a 1% solution of the most active mucoprotein fraction isolated by the ether-extraction process inhibits H.LEE to a titre of 16,000 and H.MEL to 56,000; the corresponding figures for the most active alcohol-extract from dried red cells are 8,000 and 16,000 respectively.[25] The pentane preparation, therefore, shows intermediate activity against H.LEE but is less active than those isolated by other methods against active and indicator A strains.

As would be expected, the isolated receptor-substance is inactivated after contact with living virus or with the bacterial receptor-enzymes. Inactivation by virus is slower than has been found with other erythrocyte preparations, a finding which will be discussed in detail in a later publication dealing with the interaction of virus and isolated red-cell receptor substances. The resistance to inactivation by trypsin should be mentioned here, however, since Hirst[18] stated that trypsin destroyed receptors on the erythrocyte, although in preliminary work on receptor-destroying enzymes we had not found this to be so.[7, 27] The experimental details in Hirst's paper are not entirely clear, however, no indication of enzyme concentration or time of incubation being given. In cases where trypsin enzymically inactivates receptor analogues such as sheep salivary mucoid, it does so at extremely high dilution.[28] None of the erythrocyte extracts we have prepared are significantly affected even on incubation with 1% trypsin for several hours. Howe[19] found that the antihemagglutinin activity of a "lipid-rich" stroma fraction resisted proteases, but a "lipid-poor" fraction was susceptible to chymotrypsin.

Finally, the relatively slight blood-group activity of this preparation is noteworthy, since all other erythrocyte extracts prepared in our laboratory show high activity toward the specific group from which the cells are derived, and also with regard to the heterogenetic "H" blood-group substance.[31] The specific blood-group activity of the purified pentane-treated extract is at most only 2%, and the "H" substance activity is considerably less than 1%, of the titres shown by erythrocyte preparations obtained by ether or alcohol extraction without preliminary pentane treatment (W. T. J. Morgan and W. M. Watkins, in preparation). From the data at present available it is difficult to decide whether preliminary pentane treatment degrades and inactivates blood-group substance, or whether this solvent, which appears to facilitate the release of virus receptor-substance, actually "fixes" blood-group substance to the stroma in a form which is not extractable in 50% alcohol. A similar finding with freeze-dried red-cells treated with pentane will be described in the next paper in this series. The choice of solvent used in the preliminary extraction obviously is of great importance

in attempting to obtain receptor-substance free from blood-group substance, since in no case has it proved possible chemically to separate the two substances when both are present in the one solution.

SUMMARY

A method is described for the isolation of influenza virus "receptor-substance" from human erythrocytes. Red cell stroma were shaken with pentane to form a colloidal gel from which the active substance was extracted in 50% ethanol.

A stable virus-inhibitory mucoprotein has been obtained which was completely soluble in water, 95% phenol, and diethylene glycol. The analytical figures were: N 10.0%, P 0.5%, S 2.6%, reducing sugar 14.7%, and hexosamine 9.5%. Chromatography revealed the presence of two hexosamines (probably glucosamine and galactosamine), galactose, fucose, and amino acids. Approximately 4% of ether-soluble lipid was released on hydrolysis with acid. The ultraviolet absorption spectrum suggested the presence of nucleic acid.

One very slow-moving component with only slight evidence of polydispersion was found in the ultracentrifuge. At least three components appeared on electrophoresis. After deproteinization by the Sevag technique, two sharply defined peaks were found on electrophoresis, the larger and slower-moving one alone being associated with virus inhibitory activity against indicator LEE virus.

A 1% solution of the extract inhibited hemagglutination by heated LEE virus to a titre of 10,000. The inhibition "spectrum" was H.LEE > H.MEL > H.PR8 > PR8 > LEE=MEL. After Sevag treatment approximately 80% of the activity against H.LEE and practically all activity against active and indicator A strains was removed from solution. Inhibitory activity against all strains, with the exception of MEL, could, however, be quantitatively recovered from the deposited protein-mucoprotein gel.

Capacity to inhibit virus hemagglutination was lost when the mucoprotein was incubated with active virus, or with the receptor enzyme of *V. cholerae*. Incubation with 1% trypsin did not significantly reduce the antihemagglutinin activity.

The blood-group activity of the mucoprotein was at most only a few per cent of that found in extracts prepared by other methods.

The relation of this mucoprotein to those prepared from erythrocytes by alternative methods, and to soluble "receptor analogues," is briefly discussed.

ACKNOWLEDGMENT

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PRELIMINARY PAPER ELECTROPHORETIC STUDY OF SALIVA*

Organic constituents of human saliva vary around 0.5 g. per 100 ml. depending on the nature of the stimulus and the hour of collection. Most of the organic matter is protein, with mucin being the preponderant component. However, it is one of the smaller protein elements that has been the most widely studied, mainly because of its enzymatic action, that is, salivary amylase. Phase boundary electrophoresis studies have been reported by Blix[2] on animal submaxillary mucin and by Bernfeld, Staub, and Fischer[1] and Muus[12] on human salivary amylase. In the present note, electrophoresis was carried out on whole human saliva.

The method reported here is that of zone paper electrophoresis[3, 4, 10, 17] which utilizes filter paper as a matrix to support the buffer solution. This method offers the following advantages over the phase boundary-free solution technique of Tiselius.[15]

1. By means of paper electrophoresis many determinations may be made with a volume needed for but a single sample in the phase boundary apparatus.
2. Less than 0.1 ml. samples will usually suffice for paper electrophoresis; with amounts this small, it is not possible to use phase boundary electrophoresis.
3. Optical transparency of samples is not necessary with paper electrophoresis.
4. Preparative dialysis against the buffer, which may induce degradative changes in complex materials such as saliva, is not a requirement because inorganic ions do not interfere with electrophoresis when using filter paper.
5. Separation of components by paper electrophoresis is more permanent than that produced by the phase boundary apparatus because the filter paper provides a matrix in which the protein elements are retained in their relative positions after completion of electrophoresis.
6. Enzyme migration may be traced after paper electrophoresis by testing sections of the filter paper with a suitable substrate for the specific

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** Joseph Purcell Memorial Research Fellow, Department of Surgery.

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action of the enzyme under investigation. The phase boundary apparatus is not readily adaptable to this form of enzyme determination.

7. After paper electrophoresis, the enzyme movement may be demonstrated without simultaneously showing other components which migrated to or near the same spot. Such interference often may be encountered when using phase boundary electrophoresis.

The apparatus of Köiw *et al.*[7, 10, 11] was used for electrophoresis. Acetate,

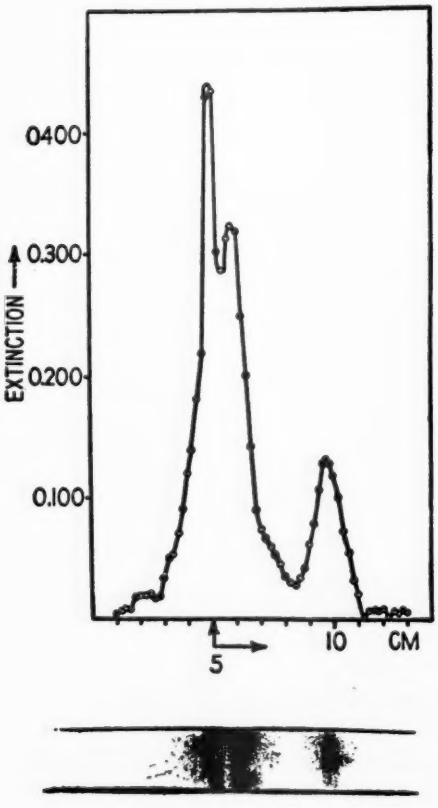

FIG. 1. Electrophoretic pattern of protein elements in 20 times concentrated human saliva. Graph (upper) and picture (lower). Arrows indicate starting point and direction of migration towards the cathode. Ordinate is Optical Density ($-\log_{10} \frac{I}{I_0}$ where I equals transmitted light and I_0 equals incident light). Run was 6 hours, 200 V, 4.2 mA, veronal buffer, pH 7.9, μ 0.05, Munktell 20 filter paper.

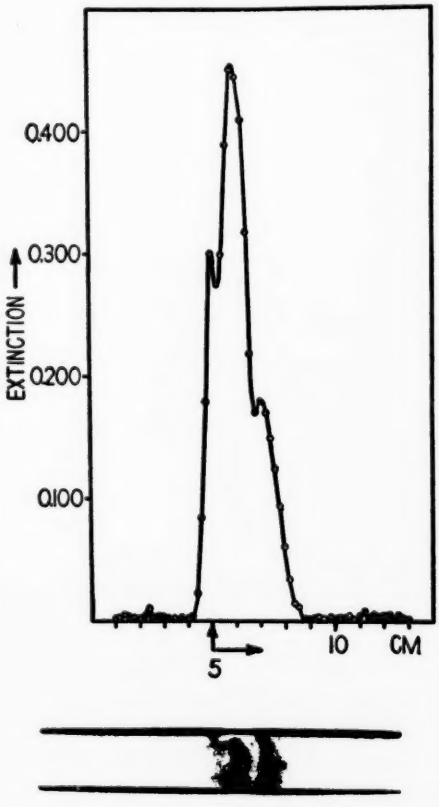

FIG. 2. Electrophoretic pattern of protein elements in 20 times concentrated human saliva. Graph (upper) and picture (lower). Arrows indicate starting point and direction of migration towards the cathode. Ordinate is Optical Density ($-\log_{10} \frac{I}{I_0}$ where I equals transmitted light and I_0 equals incident light). Run was 6 hours, 200 V, 2.5 mA, acetate buffer, pH 4.5, μ 0.1, Munktell 20 filter paper.

citrate, phosphate, and veronal buffers, pH 4.5-9.1, ionic strength 0.05-0.1, were used, along with several types of filter paper varying in weight from Whatman 1 to Munktell 20. The potential ranged from 150-300 V, currents from 1.5-5.0 mA per strip, and running times varied from 5-24 hours. Saliva samples were obtained from five healthy subjects. In some experiments, saliva was applied as secreted; in others, it was collected on ice and concentrated twenty times. After electrophoresis, the following methods were used to observe migration:

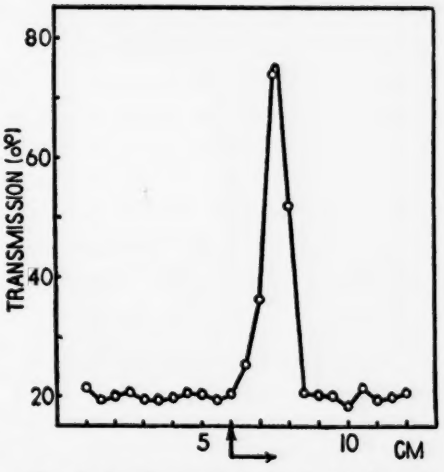

FIG. 3. Amylase activity in electrophoretic pattern of fresh human saliva. Arrows indicate starting point and direction of migration towards the cathode. Run was 8 hours, 200 V, 1.5 mA, phosphate buffer, pH 6.9, μ 0.1, Munktell 20 filter paper.

A. Protein was demonstrated with use of amido-black staining according to Grassmann and Hannig.[5,6] This method seemed more suitable than the bromphenol blue technique,[10] partly because it is more sensitive and not affected by light, and, as was reported in the case of blood serum and other protein mixtures, no correction factor is necessary for assessing protein concentration from stain intensity. After electrophoresis, dry filter paper strips were cut longitudinally in halves, and one half was stained for protein while the other half was used for one of the procedures cited below. After staining, a photometric scanning device*[8] was used to measure location and intensity of the stain on the strips. The examples in Figures 1 and 2 show the protein movement at two different pH's. Two moving fractions were noted, one moving more rapidly than the other, both towards the cathode.

B. A modified periodic acid-Schiff staining technique, suggested for blood serum by Köiw *et al.*,[9] was used for the other half of most paper strips. After staining, a considerable spot was noted at the starting point with a faint streaming toward the anode or cathode or both. The direction, extent, and intensity of the streaming varied with the buffer and pH used.

C. Position of amylase in the filter paper was demonstrated by the reduction in color of the starch-iodine reaction.[13,14] Small amounts of saliva were collected at room temperature and immediately used for electro-

* Kelab AB, Stockholm, Sweden.

phoresis. After completion of electrophoresis, wet filter paper strips were cut across into 0.5 cm. sections, each placed in a test tube containing a starch-buffer-sodium chloride solution, and incubated at 38° C. Aliquots of this digestion mixture were pipetted into another series of test tubes containing acid to arrest the enzyme activity. Finally, an iodine-potassium iodide solution was added. Samples were measured immediately in a Beckman spectrophotometer at 620 mμ. With this method, concentration of the substrate and iodine solution, and time of incubation needed to be varied depending upon the activity of the enzyme in the filter paper strips. Figure 3 is an example of amylase movement. A single peak was noted migrating toward the cathode.

By means of paper electrophoresis, it appears possible to separate protein elements in saliva. This may afford an opportunity to study differences in saliva in pathological states as compared to established norms. A more detailed description of this material will be reported later.

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THE DUAL NATURE OF ACID-FASTNESS**

Acid-fastness is usually defined by the Ziehl-Neelsen technique: staining in carbol-fuchsin, differentiation in HCl-alcohol, and counterstaining in methylene blue.[8] As a result, acid-fast substances are red, non-acid-fast materials, blue. Some authors have felt that this property of differential staining resided in one specific type of compound, the mycolic acid type of long-chain fatty acid found only in the acid-fast mycobacteria.[11] Most authorities, however, now consider that the bulk of the evidence supports a structural rather than a chemical explanation of the phenomenon. It is certainly true that grinding[16] or crushing[1] tubercle bacilli abolishes their acid-fastness although no change in chemical composition may be detected. Similar, but not identical, acid-fastness has been produced in a number of usually non-acid-fast bacteria by coating them with lipid not in itself acid-fast.[7] Spores of bacteria are usually acid-fast[9] so long as their protective capsule is present. Finally, mycolic acid, the presumed acid-fast compound in tubercle bacilli, was reexamined[19, 21] and found to be somewhat acid-fast, but not strongly enough to account for the brilliant staining of intact bacilli.

It has been recently noted[2] that under certain conditions spermatozoa are acid-fast. When an explanation of this observation was sought, certain contradictions in the basic theory became manifest. The original report of acid-fastness was by Neisser.[17] He reported that lepra bacilli, once stained, were resistant to acid destaining. This was in contrast to the ease with which other known bacteria were destained. A year later Ehrlich[10] noted a similar property in tubercle bacilli. In these latter organisms, this resistance to destaining was coupled with a resistance to staining and to chemical treatment generally.[10, 15] Out of this generalized resistance of tubercle bacilli has grown the structural theory of acid-fastness.[21] However, neither lepra bacilli[14] nor spermatozoa showed any staining resistance comparable to tubercle bacilli. It therefore appeared questionable whether the structural theory of acid-fastness as presently developed was completely valid. The object of this present paper then is to study both the differences and simi-

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larities of the acid-fast reaction in various cells and compounds, especially as the results may bear on the theory of the staining reaction.

MATERIALS

This study was made possible by the generosity of those who provided the spectrum of acid-fast materials to be tested. With the sources in parentheses, these were:

Formalin-fixed, paraffin-embedded tissue sections containing *M. tuberculosis, var. hominis,* and spermatozoa (surgical and autopsy services, Department of Pathology); *M. tuberculosis (BCG)* in guinea pig tissue and colonies of *M. ranae* (M. I. Bunting and J. Banfield, Department of Microbiology); tissues containing *M. leprae* (sent originally from Dr. G. L. Fite, U.S.P.H.S. to F. A. Putt of this department); rat liver containing *M. leprae murium* (Dr. H. S. N. Greene, Department of Pathology); mycolic and leprosinic acids (Dr. E. Mylon, Department of Pathology). Three colonies of *M. ranae* were used in this work. One was young and brilliantly acid-fast with the standard test. Smears from this colony were stained both in the intact state and after the bacilli had been crushed between glass slides. The two other colonies were old and non-acid-fast by standard tests. One was a rough and the other a smooth variant.

For uniformity, all tissues were fixed in formalin and embedded in paraffin in the usual manner. The detailed changes of acid-fastness produced by various fixatives will be described elsewhere.[6] Here it is sufficient to say that such changes are minor and occur to the same relative extent for all substances examined here.

METHODS

Unless otherwise specified, staining was done in alkaline carbol-fuchsin. Five gms. of new fuchsin (C.I. 678) and 25 cc. of phenol were added to 100 cc. of absolute alcohol. At the time of use the desired amount of this stock solution was diluted with four parts of a 1/20 M. phosphate buffer, pH 7.8. The various differentiating agents are described where used. All slides were differentiated for five minutes since this length of time had been shown to give the most reproducible results in preliminary experiments. The counterstain where used was 0.5 per cent methylene blue in 95 per cent alcohol. Mycolic and leprosinic acids were dissolved in chloroform, then adsorbed by capillary action on to strips of #1 filter paper partially immersed in the solution. This resulted in a band of acid across the strip with a sharp border between this band and adjacent control regions. Because of their thickness, the strips were differentiated for ten rather than for five minutes.

RESULTS

A. DETERMINATION OF STAINING RESISTANCE

As a baseline for subsequent work, the staining resistance of the various test materials was determined under uniform conditions. The slides and strips were stained in saturated aqueous alkaline solutions of new fuchsin (pH 7.8 as elsewhere) for 15 minutes. They were washed in three changes of 95 per cent alcohol and two changes of absolute alcohol, one minute each.

The results are given in Table 1. As was expected,[15] the various types of intact tubercle bacilli were not stained. Also as expected, lepra bacilli,[14] sperm, and crushed tubercle bacilli[1] were stained. The portions of the filter paper strips containing mycolic and leprosinic acids held more dye than the control regions.

TABLE 1. STAINING WITH AQUEOUS DYES

Material	*Stained*
M. tuberculosis var. hom.	No
M. tuberculosis, BCG	No
M. ranae ("acid-fast")	No
M. leprae	Yes
M. leprae murium	Yes
Spermatozoa	Yes
Crushed *M. ranae* ("acid-fast")	Yes
M. ranae ("non-acid-fast")	Yes
Mycolic acid	Yes
Leprosinic acid	Yes

TABLE 2. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS. NO COUNTERSTAIN.

	3% HCl 70% Alcohol	*3% HCl 95% Alcohol*	*5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	4+	4+	4+
M. tuberculosis, BCG	4+	4+	4+
M. ranae, "acid-fast"	4+	4+	4+
M. leprae	0	±	4+
M. leprae murium	0	±	4+
Spermatozoa	0	3+	4+
M. ranae, crushed	0	irregular; many 0	4+
M. ranae, "non-acid-fast"	0	0	2+
Mycolic acid	0	1+	3+
Leprosinic acid	0	1+	4+

B. RESISTANCE TO ACID-ALCOHOLS

The effects of acid-alcohol differentiations were then studied apart from the perhaps confusing effects of counterstains. The test materials were all well stained by 15 minutes in the alkaline carbol-fuchsin. Comparative differentiations were then carried out on each substance. The differentiating solutions were 3 per cent HCl in 70 per cent alcohol, 3 per cent HCl in 95

per cent alcohol, and 5 per cent acetic acid in 95 per cent alcohol.[8,18] It will be noted in Table 2 that all substances were acid-fast after acetic-acid-alcohol. By contrast, when 3 per cent HCl in 70 per cent alcohol was the differentiating agent, only those substances were acid-fast which had been resistant to staining with aqueous dyes. The results with HCl in 95 per cent alcohol fell between these two extremes.

TABLE 3. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS AND SUBSEQUENT COUNTERSTAINING

	3% HCl 70% Alcohol	*3% HCl 95% Alcohol*	*5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	Yes	Yes	Yes
M. tuberculosis, BCG	Yes	Yes	Yes
M. ranae, "acid-fast"	Yes	Yes	Yes
M. leprae	No	No	Yes
M. leprae murium	No	No	Yes
Spermatozoa	No	No	Yes
M. ranae, crushed	No	No	Yes
M. ranae, "non-acid-fast"	No	No	Irregular
Mycolic acid	No	No	Yes
Leprosinic acid	No	No	Yes

C. THE RÔLE OF THE COUNTERSTAIN

The differentiating agents used above were again tested, but with counterstaining after differentiating and rinsing in 95 per cent alcohol. Excess counterstain was removed by two rapid rinses in absolute alcohol. Table 3 presents the results of this procedure. Acid-fastness after HCl in 70 per cent alcohol was unaltered. Those materials which had retained a certain amount of dye after HCl in 95 per cent alcohol but which had been stainable with aqueous dye took the counterstain and so no longer appeared acid-fast. The only cells which took counterstain after acetic-acid-alcohol were some of the "non-acid-fast" strains of *M. ranae.* Except for this one case, the distribution of acid-fastness now paralleled that of staining resistance as given in Table 1. Those cells resistant to HCl-alcohol had also been resistant to staining. Those cells not resistant to the HCl-alcohol had been stained with aqueous dyes.

D. THE RÔLE OF PHENOL

In the above tests it was noted that crushing, which supposedly destroyed acid-fastness, altered the permeability to dyes and the resistance to HCl-

alcohols but left the organisms still acid-fast to acetic-acid-alcohol. Phenol also appeared to alter the permeability of intact tubercle bacilli to dyes, and it seemed of interest to determine whether, like crushing, the action of phenol was (i) permanent and (ii) non-specific.

Two points were reaffirmed. First, crushing had no effect upon sperm or upon already weakly acid-fast forms of *M. ranae;* it affected staining and destaining only when the cells had been stain-resistant. Second, the addition of phenol to the staining solution had no noticeable effect on the amount of

TABLE 4. ACID-FASTNESS AFTER VARIOUS DIFFERENTIATING AGENTS TO WHICH PHENOL HAD BEEN ADDED. NO COUNTERSTAIN.

	5% Phenol 3% HCl 70% Alcohol	*5% Phenol 3% HCl 95% Alcohol*	*5% Phenol 5% Acetic Acid 95% Alcohol*
M. tuberculosis, var. hominis	0*	0*	3+*
M. tuberculosis, BCG	0*	0*	3+*
M. ranae, "acid-fast"	0*	0*	3+*
M. leprae	0	0	4+
M. leprae murium	0	0	4+
Spermatozoa	0	3+	4+
Crushed *M. ranae*	0	0*	3+
M. ranae, "non-acid-fast"	0	0	2+
Mycolic acid	0	0	4+
Leprosinic acid	0	0	4+

* Differentiation altered by phenol.

dye retained under any circumstances by those cells which were stainable with aqueous dyes. These observations were then extended in the following manner. Sperm, lepra bacilli, and tubercle bacilli were treated for 24 hours with 5 per cent phenol in 20 per cent alcohol at room temperature. The subsequent staining was altered in no way by this treatment and it appeared that phenol had no permanent effect on acid-fastness; it could, however, act elsewhere than in the staining reaction. Five per cent phenol was added to each of the differentiating solutions tested above and differentiation without counterstaining was then repeated. Table 4 shows that phenol had no effect on the differentiation of any substance stainable with aqueous dyes. It did alter the stain retention of intact tubercle bacilli, however, and, just as had crushing, it made these cells slightly less acid-fast than lepra bacilli had been under the same circumstances.

A second experiment was based on the fact[18] that treatment with acids removes the acid-fastness from cells. Table 5 gives the time necessary for complete abolition of acid-fastness from tubercle and lepra bacilli in tissue sections when the sections were treated with 1 N HCl, preheated to 60° C., both with and without added phenol. Again, phenol had no appreciable effect on the reaction of lepra bacilli, but did bring about a change in the reactivity of tubercle bacilli. The generalization can thus be made that phenol, like crushing, acted where—and only where—staining resistance

TABLE 5. TIME NECESSARY FOR REMOVAL OF ACID-FAST MATERIAL FROM TISSUE SECTIONS AT 60° C.

	1 N HCl	*5% Phenol 1 N HCl 10% Alcohol*
M. leprae murium	8 min.	7 min.
M. tuberculosis BCG	12 min.	4 min.

had been present. The difference between the two means of reducing this resistance was that crushing produced a permanent alteration and phenol only a temporary one.

DISCUSSION

From the work reported here, it would appear that the arguments advanced for the structural theory of acid-fastness were valid so long as only strong acid-alcohols were used as differentiating agents. Whatever the nature of this structure, it appears that it may be altered by *prolonged* exposure to fat solvents such as xylene[13] as well as by the methods cited above. Certainly one sample of tubercle bacilli which had been thoroughly defatted during a search for soluble lipid fractions proved to have no structural acid-fastness.

The important fact, however, appears to be the suggestion that there was a second type of acid-fastness present in crushed cells as well as in intact tubercle bacilli, and present also in lepra bacilli and sperm. This second type of acid-fastness, demonstrable only after weak differentiation by such agents as acetic-acid-alcohol, was not altered by any of the mechanisms used to abolish structural acid-fastness. Further, while there was great difference between the reaction of intact tubercle bacilli and mycolic acid from these bacilli, there was little or no difference between the reaction level of crushed

tubercle bacilli and mycolic acid or between lepra bacilli and the corresponding leprosinic acid. Therefore, there appears to be support both for structural (intact vs. crushed tubercle bacilli) and "chemical" (as for mycolic acid) concepts of acid-fastness. While little needs be added to the problem of structure as treated by Yegian and Vanderlinde,[21] further study of the chemical nature of acid-fastness has yielded some interesting results. As was suggested by staining, a mycolic acid-like lipid has been found in spermatozoa.[3] Further, the reaction between mycolic acid and dye during the staining reaction has been studied and found both quantitative and unique.[4] It has then been possible to identify the same reaction in the staining of intact acid-fast cells.[5]

Granting the dichotomy of acid-fastness, it must then be decided which of the two types of reaction would best serve such purposes as the identification of mycobacteria in tissues and tissue fluids. The standard methods[8] (excepting Putt's[18]) use HCl-alcohols as differentiating agents, and so primarily demonstrate structural acid-fastness. Often rapid, incomplete differentiation is advocated so that cells such as lepra bacilli, not possessing structural acid-fastness, may yet retain the dye. An alternative method has been to provide artificial resistance by coating the cells with essential oils.[20] Since structural resistance is labile in any case, there would seem to be distinct advantages in a redefinition of acid-fastness in terms of the more stable and widespread chemical reaction.

A minor problem pointed up by this work concerns the theory of the action of phenol. Classically phenol has been held to act through the formation of a dye-phenol complex.[12] This complex was supposedly more firmly bound in the cell than the dye alone. It is not doubted that such a complex may exist in the staining solution; it is reported here, however, that phenol acts only where structural resistance is present and acts equally well during staining or destaining—it even acts to potentiate extraction of acid-fast material from the cell in a system where no dye is present. It is therefore suggested that phenol acts, not as an alum-like mordant, but to alter the structural permeability of the cell.

The present method of rapid staining at room temperature was derived from a study of Putt's[18] acid-fast technique. He attributed the accelerating action of lithium carbonate (pH 10) to a "mordanting" effect. However, it was found that any other means of providing an alkaline staining medium was equally effective, and a buffer solution was used only for convenience. Alkali, of course, had been used originally in the stain for tubercle bacilli by Koch,[15] but its value was lost sight of after phenol had been introduced by Ziehl.[22]

SUMMARY AND CONCLUSIONS

It has been shown that acid-fast materials may be considered in two groups. One group includes intact tubercle bacilli of various types; these do not stain with aqueous dye solutions but only in the presence of alcohol and phenol. Once stained, they are resistant to differentiation in hydrochloric-acid-alcohol.

The second group includes lepra bacilli, sperm, crushed tubercle bacilli, and the mycolic acids. These substances may be stained with aqueous dye solutions. They are acid-fast after differentiation in acetic-acid-alcohol but not after HCl-alcohol.

The first type of acid fastness is labile and is not only abolished by crushing, but it disappears in the presence of phenol and in old colonial forms; it fulfills the description of structural acid-fastness. The second type of acid-fastness is not labile and is altered only when the *chemical* composition of the cell is altered. It appears more suitable for diagnostic purposes than the first, structural, labile type which is shown by present methods.

Ancillary findings included: (i) counterstain may obscure acid-fastness, and (ii) acid-fast material may be rapidly stained at room temperature if the fuchsin or carbol-fuchsin is made alkaline.

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CHANGING CONCEPTS IN PUBLIC HEALTH*

"God grant us the serenity to accept the things we cannot change, the courage to change the things we can, and the wisdom to know the difference."**

Progress towards the objectives of world health and peace depends upon many factors, especially upon the understanding and wishes of the people. That public health is people is suggested in the title of a popular book.† What each person may know and do as an individual will influence much of the future of a local area and a state and a nation. Much will depend on the continuing ability of communities to send to the governing and advisory bodies of official and voluntary organizations people whose vision encompasses the health needs of the area as a whole, rather than being restricted to a group of agencies or to one particular agency. This principle, advanced by the first Director-General of the World Health Organization, Dr. Brock Chisholm, applies to the situation in the United States as well as to the future of the W.H.O. in which we are all likewise interested. This is no time for "empire building" nor for complacency.

Also necessary for progress in the improvement of health is the greater development of the skills of direction and co-ordination to ensure maximum economy and effectiveness of services. It is time for community agencies to pursue with increased vigor the establishment of long-term goals in terms of human needs, opportunities, and interrelationships. These factors deserve priority in emphasis over temporary, short-term goals, backed by skillful money raising campaigns, some of which have understandable emotional appeal. This means also that we shall do well to keep in mind the interdependence of the economic and social factors in our society. There is no doubt that gains in public health are greatly influenced by advances in other fields.

Constructive programs for public health should be dynamic and adaptable to changing needs which are influenced by conditions, duties, and occasions in a state or nation. Concepts of working together for increased effectiveness are taking many forms, depending upon the problems, on the ideas of people concerned, and on many other factors. The survey in various mani-

* Based on an address presented at the Second Annual Pennsylvania Health Conference, State College, Pennsylvania, August 17, 1953.

** From the door of a hospital for the care of chronic illness.

† Ginsberg, E. L.: *Public health is people.* New York, Commonwealth Fund, 1950.

festations, especially the community self-survey, is one illustration. It has become common practice among persons of apparent good health to visit their doctors periodically for a check-up. Similarly, in several places, for example Cincinnati and Columbus in Ohio, the "patient" (the community) has gone back to the doctor. The "doctor" in Columbus is a committee of 200 persons created for the purpose of evaluating public health services in Columbus and Franklin County. The Citizens Committee states that "re-evaluation is necessary because times greatly change needs. For example, infectious diseases are much less a menace than they were a few years ago. While they now are on the decline, the average length of life is extended. Chronic diseases and the diseases of old age have become of greater importance. We need to know how well we help with such evaluations and with research, whether we are continuing to meet the older problems of public health and whether we are taking steps to meet the newer problems." This marked change in the types of illness which are principal causes of death and incapacity requires reflection, study, and work in rehabilitation. The change also stimulates processes for joint appraisal and co-operative action. Meanwhile, the erection of a great clinical center within the structure of the National Institutes of Health will provide a new kind of approach toward the conquering of human illnesses and incapacity.

It is gratifying to see how much progress has been made beyond the so-called "basic six" services of three decades ago in the State of Pennsylvania and some of its local communities, including Philadelphia and Pittsburgh, where useful health councils and health center programs have been established. Public health activities in Pennsylvania at one time left much to be desired. Five years ago a comprehensive study was conducted by the American Public Health Association at the request of leaders in the state. This enlightening report, entitled *Keystones of public health for Pennsylvania,* was introduced by an encouraging statement and eight proposals which are now receiving continuing attention:

> Pennsylvania can enter a new era in public health. It will differ from the old by being more sound in its concepts, surer in its program, and a more intimate part of the life of the people of the Commonwealth. Though the program may be somewhat less spectacular than in the past, health workers and citizens of the new era will unite in a determined effort to:
>
> 1. Return the administration of public health to the local community;
> 2. Increase efficiency;
> 3. Complete the task of controlling tuberculosis;
> 4. Eradicate the commoner major communicable diseases;
> 5. Bring all modern resources of science to bear against rheumatic fever;
> 6. Reduce materially the ravages of cancer;

7. Attack and vigorously apply active preventive measures to the serious, ever present, and currently increasing problem of mental illness;
8. Insure the maximum application of preventive measures to safeguard the health of mothers and children, of industrial workers, and of the older citizens of the Commonwealth.

The accomplishment of these goals entails new and increasing responsibilities for the physician, still the key person in any community program, and for professional workers and taxpayers. This statement is true for all localities in these changing times.

Pennsylvania, like many other states, has great strengths and resources for its public health.* Illustrations of what a good local public health department can do, in this period of changing conditions, are being presented in many ways through various channels in the citizen effort to bring community health services up to modern standards. A nation-wide effort is going forward also to secure the provision of full-time, properly staffed and equipped local health units for the forty millions residing in several states now lacking such essential services.

Relatively little change is observed nationally in a half-dozen concepts and principles which are continuously retested and still retained:

1. The qualities of the director and of the associated individuals, within the structure of a health agency, are more significant than the pattern of any organization chart;
2. Qualities of leadership may be found in individuals belonging to a variety of cultural, economic, industrial, political, racial, religious, and other social groups;
3. Those who accept appointments to serve on boards and committees of community advisory and appraisal services do so largely because of a friendship for others accepting similar responsibilities (including the person who extended the invitation) and because of an opportunity to perform a civic duty; rarely is an interest in personal and public health the primary motive for such action;
4. Principles of sharing of tasks, of working together for a common purpose, are as old as neighborhoods, although methods differ (e.g., barn "raisings," exchanging work, sitting up with a sick neighbor);
5. People responsible for allocation of funds, within budgets, vote appropriations for services directed by persons whom they "like," and those who show reasonable returns for expenditures of time, energy, and money; an additional factor being the extent of emotional appeal;
6. Application of the Golden Rule is always appropriate.

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* Definition of public health as given to medical and public health students at Yale in 1953: Public health is the science and the art of preventing disease, prolonging life, and promoting physical and mental health and efficiency through organized community efforts for the sanitation of the environment, the control of community infections, the education of the individual in principles of personal hygiene, the organization of medical and nursing service for the early diagnosis and treatment of disease and the development of the social machinery which will ensure to every individual in the community a standard of living adequate for the maintenance of health. By C.-E. A. Winslow.

Again, on the national scene, the joining of national forces on the part of several agencies in nursing, and almost simultaneously on the part of three agencies in mental health, and more recently in New York by two agencies in blindness did not happen by accident, but by deliberate and objective steps. Now, also, the National Association for Mental Health intends to go into "large-scale" financing of research on mental illness, having previously been content to encourage research and to help initiate it through consultation with government bodies, universities, hospitals, and other research centers. Fortunately, a commission will attempt to pull together the findings of the scattered research projects now being carried on, to determine the critical spots where new research is most urgently needed, and to recommend the allocation of funds by the National Association to new or continuing projects.

Individual national agencies devoted to arthritis, cancer, cerebral palsy, diabetes, heart diseases, multiple sclerosis, poliomyelitis, and tuberculosis, for example, devote a large segment of their budgets to research, complementing efforts of governmental agencies. Before long, will there not be needed a more concerted joint educational effort for information and interpretation of results for the people who reside in our local communities and upon whom the financial and moral support of many services, in addition to their utilization, is so dependent? The agencies listed above are called popularly "health agencies," but even a superficial review of the complexity, of the ramifications, and of the results of invasion in a family of illness due to these causes indicates that the agencies of primary concern might be classed in the welfare group.

Many other questions arise for exploration and review of developments. For example, do federal budget curtailments indicate a trend toward shifting responsibility for financing health services from federal to state and local sources? Again, does the slowness in the development of full-time local health services in many areas suggest that a different program is necessary? Are there better patterns of service for metropolitan areas? Is more to be studied regarding co-operative programs of health and hospital and home care agencies through increased regionalization efforts? What of the future of policies and responsibilities of state and local health departments in meeting such problems as the use and distribution of a scarce commodity of apparently limited value like gamma globulin? What is the most effective plan for school health service? Furthermore, does the response to civil defense challenges indicate symptoms of changing concepts? Note, for example, observations of a Committee on National Emergency Services of the National Social Welfare Assembly and Community Chests and Councils

of America in July 1953: "The National defense emergency has merged with the regular economy to become the *new normality* for the nation and for social welfare. It is the continuing way of life for the forseeable future."

Have health agencies any responsibilities in relation to *housing,* including desires for new approaches in the housing of the aged? Although an increasing number of people are interested in prevention, the question of whether houses are built to live in more than to look on remains unsettled in many quarters! Many architects now are allowed and even encouraged to consider function in addition to structure. Mackintosh of London reminds us (see References) that members of housing committees should have knowledge of modern housing standards besides good will, and should consider how construction and equipment impinge upon the health and well-being of the families that live in houses built for them. He recognizes that housing is one of the four pillars of the application of medicine to man in his environment, and that the well-designed house provides opportunities for healthy living.

Are the institutions concerned with medical, dental, nursing, and other professional education prepared to assume their share of the opportunity and responsibility, whether or not there are changing concepts of public health? This is a basic question.

While the introduction of merit systems and better salaries, both on the state and local level, is becoming more common, fortunately for those interested in positions in public health, the eleven accredited schools of public health in North America preparing people for these positions are hampered by inadequate budgets and are seriously overcrowded. In addition to these ever-present problems, new conceptions of public health create other complexities and challenges. Just as years ago a specialist in the diagnosis and treatment of typhoid fever found it expedient to devote more attention to other diseases, now there are signs of new specialists in chronic illness, in skin, and in general public health activities from the ranks of venereal disease control experts.

As an example of another practical question worthy of continuing study by a group of specialists in different fields, we may consider various aspects of improvement in the soundness of the nation's financial system due to developments in medicine and public health. Are workers ill less frequently and therefore able to produce more goods? Is there better school attendance because children are in better health? What are the many factors related to recent vacancies in tuberculosis sanatorium beds in several states, and will these vacancies continue? Also, what is the effect on requirements for hospital beds and clinic care for acute diseases in contrast with what would

have been the situation were it not for the sulfonamides and antibiotics developed since 1935? What, if any, associated problems have developed? For instance, insurance policy holders are living longer, paying more premiums, and investing more money.

A quick glimpse of the recent past reminds us that in 1911, when the first full-time county health unit was established in Yakima, Washington, health administration was viewed in terms of a health officer, a nurse, and a sanitarian. Environmental sanitation and acute communicable disease control (still major lines of defense) were the primary activities of health departments. Expectation of life at birth in the general population was slightly over 50 years as compared with slightly under 70 at present, and with especially striking gains for the industrial population. At that time diphtheria, for example, flourished in New Haven and in the Territory of Hawaii, localities where there now have been no deaths from this dread disease for several years. Then, in these communities, a high proportion of mothers were attended at delivery by untrained midwives or by anyone who happened to be around, and deaths of mothers and infants were high in contrast to the present, when practically all births occur in hospitals attended by skilled physicians assisted by nurses and other trained personnel. Incidentally, in Great Britain and some other areas, where trained midwives are employed, deaths of mothers in childbirth are rare and infant mortality low, indicating that at least a partial shift back to the home for many cases of maternity and of illness might not be disastrous. The application of science to medicine and public health in the last fifty years has done more than ease suffering and prolong life; it has affected the age distribution with a relative increase of chronic diseases which are a consequence of the debilitations of the aging process.

A key to chronic disease control is early diagnosis and treatment. The opening of the new Clinical Center to expedite the attack, means, in the words of Surgeon General Leonard Scheele, "that we must not only press the search for new knowledge, but we must also use what we do know more intensively, more effectively.—Hundreds of thousands of people who will die of cancer or heart disease, or who will be permanently disabled by arthritis or neurological disorders, or mental disease, could be aided if their illness were detected in the early stages when present methods of treatment are most successful." But the Surgeon General agrees with others in the field of public health that attention must be continued for broadening knowledge of the infectious diseases, particularly of the virus infections. And, furthermore, "we have long since recognized the inseparability of science and the society in which it functions. We cannot separate a rheumatic

child's illness from his growing mind and body, from his immediate need for schooling, his family's potential need for social service. Nor can we separate the cancer patient's disease from his age, his potential unemployability, or his pension. We cannot separate the crippled workmen's paralysis from his chances for rehabilitation and return to employment and independence."

These are days when plans are being increasingly developed with participation of all concerned, based on desires and needs, scientifically tested, and carried out according to sound principles in an atmosphere of constructive human relations. Even in some of the elements of the "basic six,"* assumed to be well covered, new questions arise such as: the importance of reciprocal measures for milk control by states; development of better criteria and standards for sanitation; key points of an accelerated attack on tuberculosis; better housing for the aged and infirm; control of home accidents; the caloric diets of high nutritive quality; and the effects of changes in nurses' case loads and objectives. Sometimes it is difficult to determine wisely the activities for emphasis and to abandon old projects in order to establish more promising new ones, especially in the face of conflicting pressures and protective attitudes. It is encouraging to observe the example of representatives of the great food industry, however, in meeting with those of health departments to arrange plans for more complete and uniform safeguarding of food consumed by the American people in the home, the hospital, the industrial establishment, the schools, churches, and elsewhere.

We can adjust readily to rapid changes demanded as a result of research which provides better techniques and methods—tools with which to combat problems that threaten the health of our people. Sometimes the changes in regard to health are better understood and appreciated than so-called *public* changes. Many of us could use a more extensive knowledge of the basic social sciences, including economics and political science that might help us to understand better the people with whom we work. More and more we are beginning to recognize the importance of public changes, and are also utilizing better the possibilities for a unified attack by all members of the health team, including private physicians, dentists, and hos-

* "Desirable minimum functions" of local health departments—vital statistics and records, sanitation, communicable disease control, laboratory services, maternal and child health, and health education. Accident prevention, chronic diseases, the hygiene of housing, occupational health, school health services, mental health, medical rehabilitation, and hospital and medical care administration, besides more attention to nursing, nutrition and dental health, are other areas of service and responsibility which have been incorporated into the programs of an increasing number of local health departments.

pital administrators. Simultaneously, we find increased interest in research and in the application of results. The medical, dental, and public health schools are stimulating examples of research centers.

There is an important psychological approach also to developing principles of community organization, (as illustrated by studies of topical fluoride demonstration programs), and by developments in local and state health and hospital councils. As emphasized by Leavell (see References) and others, "The health team cannot be a closed circle of in-facing initiates with backs to the outside world; rather, it must be an open circle ready to welcome new workers and able to expand as new areas of useful cooperation are discovered."

Having in mind then the significance of the total approach with a knowledge of the individual, the family, and the community, besides the significance of the mandates of the people, we may find comfort in Edmund W. Sinnott's *Two Roads to Truth,* with a basis for unity under the great tradition. (Viking Press 1953) Sinnott reminds us that man is a creature of both mind and spirit, and destined to be the battleground between them. "He cannot abdicate the responsibilities which reason places on him, for they are what make him man. He will not sacrifice the claims of his spirit, for their authority comes from something deeper still. He is thus bound to be the seat of lifelong tension between these two contenders for his allegiance. Today he finds it difficult to reconcile the two. In the years to come he may gain wisdom enough to do so. . . . We should not regret these differences between the disciplines of reason and of spirit, but rather rejoice in them. They are the two halves that make man whole. From tension between them character is born. . . . Reason and spirit are the pillars that support our great tradition. . . . Between them they hold up the hopes of man today as he strives to fulfill his splendid destiny."

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ZOOLOGICAL JOURNAL CLUB

November 24, 1953

THE PHYSICAL ENVIRONMENT IN LAKES. By C. H. Mortimer, British Freshwater Association, Wray Castle, Lake Windermere, England.

Biological production in lakes requires both a source of nutrients and of energy. The basic problem thus becomes one of transport, of bringing to living organisms both food and the energy to utilize it, or the reverse (e.g., bringing photosynthesizing algae to the sunlit levels). The problem to be considered concerns the mechanism and manner by which mixing occurs in lakes.

During the spring, a time when there is rapid heating of surface layers, the upper layer is heated to a temperature above 4° C. (4° C. is the temperature at which water is most dense) and rises and spreads over the surface with the consequent production of a density gradient. When a wind blows across the lake, a velocity gradient which is opposite in direction to the curve of the density gradient, is superimposed upon the latter. Density increases with depth and velocity of movement decreases. The force which tends to increase the resulting turbulence is the wind, while the density gradient tends to suppress this. The criterion which determines whether turbulence will increase or not is Richardson's number or

$$\frac{(dv/dz)^2 \text{ (which represents the velocity gradient, or shear)}}{(dv/dz) \text{ (which represents the density gradient)}}.$$

If this value is greater than unity, turbulence will increase, while it will decrease if it is below unity. If the surface layers have an Ri greater than one and the lower levels have a value less than one, stratification results. In the autumn, with rapid cooling of surface layers, the same conditions exist.

As a consequence of a wind blowing across a small lake where the momentum of the earth's rotation can be neglected, there is a piling up of water to the windward side. Bottom water consequently upwells to the lee and the return current from the windward upwells at the center. When the wind ceases, there is a seesawing oscillation of the various layers with much horizontal movement. The result is a breakdown of the stratification which may later be re-established.

In larger lakes there is superimposed on these movements a seesawing in three planes due to the force of the earth's rotation. This may considerably modify the other movements, depending on the degree to which the various movements are in or out of phase.

The studies which demonstrated these phenomena were made in a number of glacial lakes near the British Freshwater Association's Wray Castle station. Additional studies were carried out in the laboratory using model lakes in which vacuum machine motors served to create wind, liquids of different densities provided stratification, heated wires substituted for solar energy, while dyes and dispersed crystals of permanganate enabled observations of the water movements to be made.

P. K.

ZOOLOGY-BOTANY JOURNAL CLUB

December 2, 1953

MACROEVOLUTION AND THE PROBLEM OF MISSING LINKS. By Alexander Petrunkevitch, Professor Emeritus of Zoology, Yale University.

In this discussion we shall be concerned with general theory rather than details. It is not that these are lacking, but that they are of so specialized a nature as to be the best antidote for insomnia.

Darwin's assumptions regarding the origin of species included the postulation of intermediate links. If speciation is the only method of evolution, it should not be impossible to find fossil remnants of some of these transitional forms. However, they have still to be demonstrated. In addition, we are faced with the problem of there not having been enough time to account for the wide divergencies that have arisen. This remains true even when we assume a new species to be formed by a single mutation, an unlikely occurrence at best. Finally, in order for speciation to produce new orders or classes, we would have to assume that dedifferentiation had taken place, i.e., we would expect to find regressive links showing a loss of specialized structures. This would involve even more time, which has simply not been available. Many modern examples of generic and family speciation exist with connecting links present. Transitional forms between groups of the ordinal level and above, however, have only been inferred by vertebrate paleontologists from their studies of comparative anatomy.

We suggest that the absence of these transitional forms is due to evolution having proceeded by way of gross changes. Geneticists object that sudden major changes are generally lethal, but we have seen viable human monsters produced not infrequently. Similar phenomena are to be found in all groups of animals and these creatures can survive and reproduce. Macroevolutionists, then, believe in the sudden appearance of "hopeful monsters," which might sometimes have adaptive advantages. In addition, they would not need to submit to dedifferentiation as would be true if evolution proceeded by speciation.

How can we recognize evidences of macroevolution if there are no intermediate forms? Obviously, such nonexistent forms will not be found. The evidence can be seen only by examination of the over-all trend shown by the particular organism. We postulate that, following the coalescence of chemical substances to form protoplasm, the first living animal was a gastrea—an organism of the structure of a simple gastrula. This inturn produced a number of different forms, each of which was destined to give rise to a separate phylum. Evolution, then, proceeded separately within each phylum by way of further macroevolutionary patterns.

P. K.

BOOK REVIEWS

TEXTBOOK OF CLINICAL PARASITOLOGY. 2d ed. By David L. Belding. New York, Appleton-Century-Crofts, Inc., 1952. viii + 1,139 pp. $12.00.

Ten years ago Dr. Belding brought out the first edition of this text and in the present volume follows the original plan of presenting "the protozoan, helminthic, and arthropod parasites of man, and the diagnosis, treatment, and prevention of the diseases they produce." Although more material has been compressed into each page and thinner paper permits a convenient reduction in its thickness, the book is actually about 250 pages longer than the first edition.

In almost every chapter, sections on diagnosis, treatment, methods of laboratory diagnosis and cultivation, nutrition, immunology, and reproduction are expanded. Chapters on amebiasis, the blood and tissue flagellates, malaria, the filarioidea, and schistosomes are especially notable for the choice and amount of new material included. The toxoplasma are assigned a separate place for the first time. The technical section is larger; parasitic protozoa, helminths, serological and chemical methods, and intermediate and reservoir hosts each occupy a chapter. About thirty new drugs, complete with structural formulae, contraindications for use, and methods for testing are added.

This is both a reference and a textbook. Tables, graphs, photographs, diagrams of life cycles, and extensive line drawings of ova and parasites facilitate its use; each succinctly illuminates the writing. The designer of the book uses type faces which eliminate any confusion in finding appropriate sections; this is a striking improvement over the first edition. An annotated bibliography is included for the first time; a marked increase is noted in the number of references. In this edition the author has achieved his purpose and presents us with a more handsome, complete, and useful book.

J. K. R.

SOVIET SCIENCE. Edited by Ruth C. Christman. Washington, American Association for the Advancement of Science, 1952. vii + 108 pp. $1.25.

This short book is a good bock which consists of nine papers read at the 1951 meeting of the American Association for the Advancement of Science. Among the papers are "Russian Genetics" by Theodosius Dobzbansky, "Russian Physiology and Pathology" by W. Horsley Gantt, "Russian Psychology and Psychiatry" by Ivan D. London, and "Russian Contribution to Soil Science" by J. S. Joffe. Several of the authors were born and trained in Russia while others have made one or more professional visits to the country. It is apparent that each author has a significant degree of knowledge and understanding of Russian scientific activity in his particular field.

The purpose of *Soviet Science* is to present a factual report of the current position of various sciences in Russia, and it achieves its purpose admirably. The sources of the reports consist largely of the authors' personal knowledge and current Russian scientific literature which Howard A. Meyerhoff correctly points out in the Foreword is "much more . . . available on this side of the Atlantic than the average American may think."

The importance of the book extends beyond informing Western scientists of the activities of Russian scientists. For instance, it is abundantly clear, although *Soviet Science* does not say so in as many words, that as long as Lysenkoism persists in Russia, the economic and consequently the military potential of the U.S.S.R. will be lessened due to the deleterious effect of Lysenkoism on agriculture. It is also important to know that Russian mathematics is excellent.

It seems to this reviewer that Conway Zirkle is accurate in concluding "An Appraisal of Science in the U.S.S.R.," the last paper in the volume, with the following statement. "We may characterize Russian science by paraphrasing the description of the little girl we learned about in kindergarten 'When it is good, it is very, very good; but when it is bad, it is horrid.'"

FREDERICK G. KILGOUR

STUDIES IN PATHOLOGY PRESENTED TO PETER MACCALLUM. Edited by E. S. J. King, T. E. Lowe, and L. B. Cox. Melbourne, At the University Press, 1950. x + 350 pp. 35/—.

This collection of papers is a *Festschrift* to Professor Peter MacCallum, on his sixty-fifth birthday, marking over a quarter of a century of service and association with the University of Melbourne. The subjects included are diverse enough, though about twenty of the twenty-three papers are more or less directly concerned with anatomical pathology, but the individual papers are so specialized that the volume, as a whole, will probably have a limited appeal. Contributions and contributors are too numerous to list here. The book is indexed.

I found the papers by E. S. J. King and R. A. Willis especially stimulating. Pathologists are bound to find the book thought-provoking and worth their while. Others will find individual papers of interest. I would advise those in the medical field to make a point of browsing through this book in a library to look for their special interests. This book has its place in medical and pathology libraries, but probably does not belong in a general library.

J. M. Q.

NUTTALL'S TRAVELS INTO THE OLD NORTHWEST. Jeannette E. Graustein, Ed. Published as No. 1/2 of Vol. 14 of The Chronica Botanica Co., Waltham, Mass.; New York, Stechert-Hafner, Inc., 1951. 88 pp. illus. Paper $3.00.

Thomas Nuttall, native of Yorkshire, England, came to Philadelphia in 1808 at the age of twenty-one. Almost immediately he made the acquaintance of Benjamin Smith Barton, M.D., Professor of Materia Medica, Natural History, and Botany at the University of Pennsylvania. Because of his youth and devotion to the study of Botany, Nuttall was hired by Barton for extensive field trips and the collection of specimens.

Two earlier trips, one to Delaware, where Nuttall contracted malaria, and one to Niagara Falls and Ontario paved the way for the travels recounted in this diary. Nuttall's 1810 expedition, the first months of which are described in the diary, was unquestionably undertaken for Barton at his expense. The route, objectives, and full terms of agreement were set down in detail by Barton. Nuttall, completely ignorant of frontier conditions, unable to swim or shoot, proposed to travel through the vaguely known territories far to the North West. Only three and a half years after the return of the Lewis and Clarke Expedition, with English military posts and fur companies unfriendly, and many of the Indian tribes hostile to the Americans, Nuttall had little idea of the enormity of his commission, and a record of his first day's observations breathes confidence and enthusiasm.

From Philadelphia he proceeded by coach to Pittsburgh, then afoot through Franklin, Waterford, and Erie, and along the south shore of Lake Erie as far as the Huron River; from here by boat to Detroit, and then by canoe to "Michilimakinak." There are dated records from the banks of the Fox River and at the portage to the Wisconsin. Later records are from St. Louis, and also after his return from the upper Missouri.

Nuttall was able to proceed with the western-most part of his trip by joining the Astoria expedition, on their way to establish fur trading posts on the Columbia River. He separated from the Astorians when fear of the Blackfeet Indians made them abandon the river route. With rich collections Nuttall returned to St. Louis. He then traveled by boat to New Orleans and because of impending war he sailed back to England, sending notes and specimens to Dr. Barton.

The subject matter of the diary is chiefly biological, with description of plants, animals, and lists of plants and some of animals. Rocks, oil, and mineral springs, and especially Indian mounds are discussed. There are occasional notes on towns and topography: "To day I passed thro' a sandy plain covered with the ripe fruit of the Fragaria, & alive with snakes as the day was warm. I came to a small settlement on Kiaoga called Cleveland where I spent the remainder of the day." There are notes on goitre and other miscellaneous interests of Dr. Barton. Nuttall made other journeys into western United States. These are not, however, covered by this diary.

J. J. C.

A History of Medicine. Volume I: Primitive and Archaic Medicine. By Henry E. Sigerist. New York, Oxford University Press, 1951. xxi + 564 pp. $7.50.

Henry Sigerist is the dean of historians of medicine. Since his resignation as Director of the Institute of the History of Medicine at Johns Hopkins University, Sigerist has been working on the culmination of his life's work, an eight-volume history of medicine of which this volume is the first. Its introduction is an excellent 100-page exposition of Sigerist's concept of the history of medicine. The other three sections are "Primitive Medicine"

which includes the medicine of primitive peoples today, "Ancient Egypt," and "Mesopotamia."

A remarkable linguist, Sigerist has used original documents most extensively. Because of language barriers, few other historians in any field could employ as wide a range of sources as Sigerist has. Moreover, he has interpreted his sources in a truly historical manner. Most histories of medicine are of the "cut-and-paste" variety which treat medicine as an activity having no relation to history in general. Not so this volume. Sigerist discusses developments in medicine as part of man's social and intellectual history. Although it has been said with justification concerning Sigerist's earlier works that "the Marxist interpretation of history appeals very much to him," there is no Marxist interpretation in this volume.

No other work on medicine in Egypt and Mesopotamia approaches this volume in either completeness of coverage or excellence of historical interpretation. When Sigerist has completed the remaining seven volumes, he will have made an unequalled contribution to the history of medicine.

FREDERICK G. KILGOUR

THE LIVING BRAIN. By W. Grey Walter. New York, W. W. Norton and Company, Inc., 1953. 311 pp. $3.95.

The problem of an author who wishes to popularize advances in science is a delicate one. He must use analogies and simplifications which are clear and interesting to the lay reader, and which at the same time remain faithful to the facts of the scientist. One trap is the risk he runs of boring the intelligent but uninformed reader, on the one hand with countless tables of data, esoteric discussions, and pages of evidence, or on the other with condescension in style and presentation. A second trap is that of abusing the experimental worker's findings by oversimplification in an attempt to be lucid. The writer must be true to both the layman and the scientist.

In *The Living Brain,* W. Grey Walter has succeeded in a popular presentation of the previous developments and recent advances in neurophysiology. The material is interestingly written. His fine discussion of the brain's alpha, delta, and theta activities and their correlation with personality is especially well stated, as are his review of our developing comprehension of the learning process and his section concerning the remarkable effects of flickering light on the brain waves and psychology of susceptible subjects. In the context of these very readable chapters he can be forgiven his occasional lapses into the maudlin (e.g. in discussing Devonian animals he says that "there is something endearing about the early monsters") or the obscure (of the sponges' agglomeration after being put through a sieve: "If their resurrection is symbolic, their way of association is a model for the anarchist and their inter-communication a gift to telepathy.")

The book does not pretend to give solutions, but it offers a review of a new approach to the mind-body problem which has perplexed philosophers and scientists for centuries.

G. A. F.

STUDIES ON DRIED BCG VACCINE. Tokyo, The Japan BCG Research Council. 99 pp.

This report, including an abundance of tables, describes in detail various experiments designed to throw light on problems encountered in the Japanese program of producing BCG vaccine for immunization against tuberculosis. Studies were made of production of dried vaccine, of methods of preservation, of virulence, and of potency in guinea pigs. Discussion follows the description of each experiment, so that the whole is essentially a series of journal papers. The "studies" will be of interest only to the production man, and he will have read their counterparts elsewhere.

PHILIP B. COWLES

MORRIS' HUMAN ANATOMY. Edited by J. Parsons Schaeffer. 11th ed. New York, Blakiston, 1953. xiv + 1718 pp. $16.00.

The eleventh edition of this extensive, time-honored textbook of human anatomy constitutes a complete revision of the previous one in that every section has been rewritten and brought up to date. Three hundred and sixty new or radically revised illustrations are included. Although developmental anatomy is in a major sense considered in a separate section, helpful embryologic explanations and illustrations are included at pertinent places in each section. Throughout the text, reference is made at appropriate locations to clinical aspects and relations which increases the use of the book as a practical reference as well as a text. While each of the fifteen able contributors is primarily responsible for the preparation and scope of the subject-matter of his assigned section, the editor has done a good job in co-ordinating and integrating the whole work to make it as uniform and complete as possible. The Blakiston Company, which has published every edition of the text since 1897, continues to produce a clearly printed and attractive book.

E. S. CRELIN

RUDOLF VIRCHOW; DOCTOR, STATESMAN, ANTHROPOLOGIST. By Erwin H. Ackerknecht. Madison, University of Wisconsin Press, 1953. xv + 304 pp. $5.00.

In 1948, the government of the Russian zone of Germany issued a series of stamps commemorating great German socialists such as Marx and Engels. The 16 pf. and 25 pf. stamps of this series have a portrait of Rudolf Virchow (1821-1903). In 1877 at Munich, Virchow spoke against Haeckel's proposal that evolutionary biology should be the foundation of all education. Virchow's criticism of Haeckel's scientific theories was sound, but his statements about education were very unsound. He appears to have gone so far as to suggest that Darwinism was akin to socialism, a suggestion which in 1877 in Germany was the equivalent of saying today in the United States that biological evolution and communism are alike. As an indirect result of Virchow's pronouncement, the teaching of biology was excluded

from the higher classes in the schools. But previously, in 1848, Virchow had fought on the side of the revolutionaries. What manner of man was this who seems to have been on both sides of the issue?

Erwin Ackerknecht, Chairman of the Department of the History of Medicine at the University of Wisconsin, states "that during the last twenty years the facts of his [Virchow's] life and work have become more and more submerged by a mostly negative 'myth.' " Certainly the Russian authorities in East Germany have contributed to the myth. However, Ackerknecht in his excellent biography, the first in English and the first of real competence in any language, has presented the facts which should go far toward elucidating the real character of the man.

Virchow was a small, dynamic person with a prodigious capacity for productive work in pathology, politics, and anthropology; J. Schwalbe's *Virchow Bibliographie,* which excludes his political writings, contains more than 2,000 items. In this biography, Ackerknecht has made wide use of Virchow's writings but points out that Virchow's personal archives were unavailable, being in East Germany. Therefore, Ackerknecht has concentrated more on the man's work than on his personal life. He divides his book into four sections: "Rudolf Virchow: Life History," "The Doctor," "The Statesman," and "The Anthropologist."

As already pointed out, this book is the first full-length biography. It not only adds much to our knowledge and understanding of the man who produced the concept of cellular pathology, but also makes a significant contribution to the history of biology in the last half of the nineteenth century.

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*Adams, C. H., and Cecil, R. L.: Ann. Int. Med. *33*:163, 1950.

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